

STEROID RECEPTORS IN HUMAN ENDOMETRIAL AND BREAST CANCER, AND, BINDING SITES FOR TAMOXIFEN AND ESTRAMUSTINE

**A study on analytical procedures
and on clinical correlations**

Mårten Fernö



LUND 1985

**STEROID RECEPTORS IN
HUMAN ENDOMETRIAL AND BREAST CANCER,
AND, BINDING SITES FOR
TAMOXIFEN AND ESTRAMUSTINE**

**A study on analytical procedures
and on clinical correlations**

Mårten Fernö

**Department of Oncology, University Hospital, S-221 85 Lund,
Sweden**

Lund 1985

To My Family

The present thesis is based on the following papers as well as hitherto unpublished results, which will be presented.

- I. Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer.
Norgren A., Borg Å., Fernö M., Johansson U., Lindahl B., and Tsiobanelis K.
Anticancer Research 2: 315-320, 1982.
- II. A comparison between two steroid receptor assays in breast cancer: dextran coated charcoal and isoelectric focusing.
Fernö M., Borg Å. and Norgren A.
Anticancer Research 3: 243-246, 1983.
- III. Observations on wet weight, protein and DNA as reference for steroid receptors in malignant mammary tumours.
Norgren A., Fernö M. and Borg Å.
Accepted for publication in Anticancer Research, 1985.
- IV. Prognostic significance of estrogen and progesterone receptors in stage II breast cancer.
Rydén S., Fernö M., Borg Å., Hafström L., Möller T. and Norgren A.
(South Swedish Breast Cancer Group)
Submitted for publication.
- V. Plasma steroid hormones, cytosol receptors and thymidine incorporation rate in endometrial carcinoma.
Lindahl B., Alm P., Fernö M., Norgren A. and Tropé C.
American Journal of Obstetrics and Gynecology, 149: 607-612, 1984.
- VI. Antiestrogen binding sites in human breast cancer biopsies. Measurement, ligand- specificity and -affinity, and correlation to estrogen and progesterone receptors.
Fernö M. and Borg Å.
Anticancer Research, 5: 307-312, 1985.
- VII. Estramustine binding site in human breast cancer biopsy samples. Its relation to estrogen and progesterone receptor levels, age and menopausal status.
Fernö M., Borg Å. and Idvall I.
Submitted for publication.

References to these papers will be made by citation of the appropriate Roman numerals.

CONTENTS

ABBREVIATIONS.....	9
INTRODUCTION.....	11
AIMS OF THE STUDY.....	23
MATERIAL AND METHODS	25
RESULTS.....	31
Paper I.....	31
Paper II.....	32
Paper III.....	32
Paper IV.....	34
Paper V.....	36
Paper VI.....	37
Paper VII.....	38
DISCUSSION.....	41
SUMMARY AND CONCLUSIONS.....	53
ACKNOWLEDGEMENTS.....	55
REFERENCES.....	57

PAPERS I-VII

ABBREVIATIONS

AEBS _{spec}	specific antiestrogen binding site
AEBS _{tot}	total antiestrogen binding sites
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
DCC	dextran-coated charcoal
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetat
EMBP	estramustine binding protein
EMBS	estramustine binding site
EMP	estramustine phosphate
ER	estrogen receptor
fmol	femto(10^{-15})mol
g	relative centrifugation force
IF	isoelectric focusing
K _d	dissociation constant
M	moles per litre
MD	moderately differentiated
N-d-Me-TAM	N-desmethyl-tamoxifen
ODU	optical density unit
4-OH-TAM	4-hydroxy-tamoxifen
PD	poorly differentiated
PgR	progesterone receptor
RBA	relative binding affinity
RFS	recurrence free survival
r _s	Spearman's rank-correlation
S	Svedberg unit
SD	standard deviation
SDG	sucrose density gradient centrifugation
TAM	tamoxifen
TRIS	tris(hydroxymethyl)aminomethane
WD	well differentiated
w/v	weight per volume
$\bar{x}$	mean value

INTRODUCTION

HORMONES

The endocrine system, like the nervous system, adjusts and correlates the activities of the various body systems according to the changing demands of the external and internal environment. This adjustments are brought about by the secretion of hormones, chemical agents produced by ductless glands and passed into the circulation to regulate the metabolic processes of various cells. The birth of the science of internal secretion dates back to 1849, when the German physiologist Arnold Berthold reported on the restitution of cock characteristics by the implantation of testes into capons. He inferred that the testes produced a substance that was released into the blood stream. The term hormone was introduced in 1904 by W.M. Bayliss and E.H. Starling to denote in general the products of internal secretion that are distributed by the blood.

Hormones, at least hormones in the classical sense, are generally accepted as exerting their action by some kind of receptor. Hormones may be divided into two groups on the basis of solubility and mechanism of action (Williams, Textbook of Endocrinology, 1981).

1. Most water soluble hormones, such as peptide hormones and catecholamines, interact with receptors attached to the cell surface (Catt and Dufau 1977). This interaction activates a transmembrane messenger, which in most cases results in an activation of adenylcyclase. Adenylcyclase converts then ATP to the second messenger cAMP (Haynes, Sutherland and Rall 1960), which activates enzymes, such as protein kinases (Williams, Textbook of Endocrinology, 1981).

For a minority of hormones in this group, (eg. insulin, prolactin and growth hormone), other intracellular second messengers with similar mechanisms have been postulated (Goldberg and Haddox 1977). Furthermore, receptors for insulin, prolactin and some other polypeptide hormones have also been detected in other membranous structures of the cells, including the rough endoplasmic reticulum, Golgi apparatus and the nuclear membrane (Gorden et al. 1980, Rillema 1980).

2. Lipid soluble hormones such as steroids (glucocorticoids, mineralocorticoids, estrogens, gestagens and androgens), the sterol 1,25 (OH)₂ vitamin D and the thyroid hormones cross the plasma membrane, probably by passive diffusion. These hormones have their receptor inside the cell and the nucleus is their major site of action (Gorski and Gannon 1976, Sterling 1979).

The first significant advance for an understanding of the chemical basis for endocrine responses, especially for estrogens, was the isolation of estrone in urine from pregnant women and from stallions, by Doisy in 1929. Some years later, 1935, MacCorquodale, Thayer and Doisy extracted a substance from pig ovaries, which later was identified as estradiol (MacCorquodale et al. 1936). In 1959 Glascock and Hoekstra synthesized tritium labelled hexaestrol and after injection in goats it was concentrated and retained in the uterus and vagina, organs known to be target organs for estrogens (Glascock and Hoekstra 1959). The synthesis of ³H-estradiol with high specific activity made it possible to confirm the findings by Glascock and Hoekstra (Jensen 1960, Jensen and Jacobson 1960, 1962). Some years later Jensen, DeSombre and Jungblut (1967) and Terenius (1968), independently demonstrated the specific binding of radio-actively labelled estradiol in some samples of breast cancer. The accumulation of steroids in target organs was later considered to be due to specific binding proteins, called receptors (Jensen et al. 1971).

Mechanism of action. The hitherto generally accepted mechanism for steroid hormone action, involves the passive diffusion of the hormone through the plasma membrane and an interaction of the steroid with cytoplasmic water soluble receptor protein. The steroid-receptor complex then undergoes a temperature dependent conformational change (receptor transformation) after which the complex is translocated to the nucleus (receptor translocation), resulting in the binding to acceptor sites on the chromatin, gene transcription and altered protein synthesis (Gorski et al. 1968, 1976, Jensen et al. 1968, 1972, 1973).

Alternative mechanisms of action can however not be excluded. Binding sites for estrogens, similar to the receptors, have been demonstrated on the cell surface (Pietras and Szego 1979, Zänker et al. 1981) as well as microsomal binding sites (Parikh et al. 1980). In addition, a further cytoplasmic binding site for estrogens, the so called type II receptor, has been discovered, first in rat uteri and then also in breast cancer (Clark et al. 1980, Panko et al. 1981). The importance of the type II binding site is not yet established, but it is suggested to be involved in the retention of estrogens, thus creating an estrogen-rich environment for the type I site (ER). Alternatively, the type II site may represent a type I precursor (Clark et al. 1980).

Recently two reports (King and Greene 1984, Welshons et al. 1984) have suggested that cytosol as well as nuclear forms of the receptor protein, may in the intact cell reside in the nuclear compartment.

Steroid receptor criteria. It is now generally accepted that the presence of a biologically significant steroid receptor in a given tissue must fulfil the following criteria (Clark et al. 1976, McCarty et al. 1980):

1. Saturability (limited binding capacity at a blood hormone concentration of approximately 10^{-10} - 10^{-8} M).
2. Specificity for a given class of hormones.
3. Distinct molecular weight.
4. Correlation between the receptor content and the biological response of the steroid.
5. Tissue specificity (500-20,000 binding sites in target cells and almost absent in non-target cells (Wittliff et al. 1980)).
6. High affinity binding, with dissociation constants in the order of 0.1-1.0 nM.

Measurement of estrogen (ER) and progesterone receptors (PgR). During the last 10-15 years a large number of techniques for the measurement of cytoplasmic ER and PgR have been developed. For review see King (1975), Wittliff (1975), Clark et al. (1976), Chamness and McGuire (1979) and Seibert and Lippman (1982). The preparation of cytosol is performed by a homogenization of tissue material in buffer, followed by a centrifugation at, usually 100,000xg. The thereby obtained supernatant is referred as the cytosol.

Most of these methods are based on the ability of steroid receptors to form saturable complexes with radio-labelled natural hormones or synthetic ligands. Besides a quantification, some characteristic property is usually also studied in order to distinguish receptors from one another and from other hormonal binding sites.

The most common methods worldwide for ER and PgR analyses, are the multiple point dextran-coated charcoal (DCC) assay with Scatchard analysis (Korenman 1968, Korenman and Dukes 1970, Feherty et al. 1971) and sucrose density gradient centrifugation (SDG; Toft and Gorski 1966, Jensen et al. 1971). With these techniques, the hormone receptors are characterized by their dissociation constants (K_d) and their sedimentation characteristics (Svedberg unit = S), respectively. By using isoelectric focusing, the receptors are measured at their isoelectric points (Wrange et al. 1978).

A consensus development meeting on "Steroid Receptors in Breast Cancer", held in the USA at the National Institute of Health on June 27-29 1979, has recommended that the most reliable and reproducible methods for cytosol ER assay, appear to be SDG and DCC method with Scatchard analysis (DeSombre et al. 1979). It was also suggested at the meeting that other techniques should be validated against these methods.

At our laboratory, the DCC assay with Scatchard analysis and isoelectric focusing have been the routine methods for receptor (ER and PgR) measurements and in the following only these two techniques will be considered.

The DCC assay, originally described by Korenman (1968), is based upon the incubation of cytosol with increasing concentrations of radioactive ligand to saturation, in the absence and presence of unlabelled ligand competitor. Excess free ligand is absorbed by dextran-coated charcoal. The incubation with unlabelled ligand is made in order to correct unspecific binding. Scatchard analysis (1949) or similar methods are used in order to quantify the number of binding sites and to obtain the dissociation constant (K_d) for the hormone-receptor complex.

Some disadvantages with the DCC technique may be mentioned: a/ The demand for comparatively large amounts of tissue, b/ possible interference with other non-receptor binding proteins (McCarty et al. 1980), c/ cross reaction with other receptors (Lippman et al. 1977), and d/ the separation between specifically and non-specifically bound ^3H -ligand is made with charcoal, which has been reported to reduce receptor (Ralet and Brombacher 1981) and protein (Hähnel and Twaddle 1979) recovery.

Isoelectric focusing (IF), with the use of polyacrylamide gel, allows the separation of free and non-specifically bound ligands from specifically bound ones, on the basis of the charge property of receptors at different pH (Gustafsson et al. 1978, Wrangé et al. 1978). IF can be used for ER as well as for PgR assays and both receptors can also be analyzed simultaneously in the same gel (Wrangé et al. 1981). In a comparison with SDG, IF was found to be a more sensitive technique (Wrangé et al. 1976). Only one incubation per sample is needed, which means that smaller amounts of tissue suffice for a reliable analysis (Wrangé et al. 1981). This has also made it possible to measure receptors (mostly ER) in fine needle aspirates (Lindgren et al. 1980, Silfverswärd et al. 1980). Problems inherent in this method are those usually encountered with other types of electrophoresis, such as receptor aggregation and interaction of receptor with reactants in the gel (Seibert and Lippman 1982).

Besides the analytical steps involved in the different methods mentioned above, there are several other methodological points that should be mentioned, which are in common for all methods and are of great importance for the reliability of the receptor measurements. As ER and PgR determinations are used in clinical practice for the evaluation of breast cancer patients, most methodological knowledge has been obtained from breast cancer samples.

Hormone receptors are heat-labile proteins and tissue should therefore be

chilled and kept at 0-4°C immediately after surgical removal of the biopsy sample and then put, as quickly as possible, into liquid nitrogen (or -70°C). Different studies have demonstrated that the half-life of ER and PgR are highly variable in intact biopsies, ranging from as little as 30 minutes to no change in six hours at room temperature (Wittliff 1984). A general rule is that the tissue should be frozen 30-45 minutes after surgery and preferably maintained on ice or in a refrigerator before freezing (Wittliff 1984).

After specimen collection, the following steps in the analysis of receptors involve thawing, homogenization in buffer and homogenate centrifugation. Many different procedures have been reported, which may have an influence on the assay. Besides being heat-labile, steroid receptors are unstable proteins, dependent on pH and ionic strength. During the assays it is therefore necessary to keep the samples at 0°C. The importance of homogenization technique, choice of buffer and also pH in the cytosol are indicated in different investigations (Chester et al. 1975, Witliff 1975, Norgren et al. 1985). In order to increase the stability of the receptor, sulfhydryl-protecting compounds such as dithiothreitol (McGuire and DelaGarza 1973, Rice et al. 1982) or mercaptoethanol (Braunsberg et al. 1975) can be added. Other possible reactants in the buffer are molybdate (Anderson et al. 1980), protease inhibitor (Vass and Green 1979), KCl (Daehnfeldt and Briand 1977) or glycerol (Feil et al. 1972, Young and Cleary 1974). The centrifugation of the homogenate is usually performed at 100,000xg for one hour, but other g-values have been used (Chester et al. 1975). Furthermore, the recovery of receptors is dependent on the protein concentration, which should be kept not lower than 1 mg protein per ml supernatant (Garola and McGuire 1978, King et al. 1978, E.O.R.T.C. 1980). Powell et al. (1981) have reported reliable results down to a protein concentration of 0.7 mg/ml, if bovine serum albumin was added to the charcoal solution prior to use.

The presence of non-receptor binders such as albumin, corticosteroid-binding globulin and sex hormone-binding globulin, which are present in tumour specimens may also influence the assays (Seemater et al. 1978, Chamness and McGuire 1979, Seibert and Lippman 1982). The choice of ligand (Raynaud 1977, Raynaud et al. 1978), the incubation time (Chester et al. 1975, Martin et al. 1978, Raam et al. 1981), the incubation temperature (Raam et al. 1981) and the time of dextran-coated charcoal treatment (Ralet and Brombacher 1981) have also been reported to be analytical steps of importance for the receptor recovery.

From what has been said above, it is evident that for the reliable use of steroid receptor measurements in the clinical management of breast cancer

patients, there is a great need for methodological standardization as well as improvement and also for quality control of receptor assays, both within the same laboratory and between different laboratories (Wittliff 1975, Raam et al. 1981, Koenders and Thorpe 1983 I and II). It seems also desirable to develop new methods not based upon the hormone receptor binding capacity, which might contribute to the development of a technique more easily to standardized. An example is the recently developed ER enzyme immuno assay procedure, based on direct antigenic recognition of the receptor (Greene and Jensen 1982).

Another question concerns the best way to express the receptor concentration. Protein is hitherto the most commonly used reference parameter. Other possible parameters are tissue DNA (Silfverswärd et al. 1980) and wet weight (Jensen et al. 1976). As the malignant epithelial cells are present in variable proportions, together with normal epithelial cells, stromal tissue, fat, and in some tumours an infiltration of lymphocytes; the presence of these non-malignant cells will influence all the three reference parameters mentioned above. A correction based on the actual amount of carcinoma cells present, has been reported and considered to be of clinical significance (Gairard et al. 1981, van Netten et al. 1982).

After the quantification of receptors, a further question is the definition of the cut-off point for distinguishing between receptor positive and receptor negative samples. This value depends on the sensitivity of method used by different laboratories and, usually, lies between 3 and 10 fmol/mg supernatant protein. Values in this interval is then, in most laboratories, applied in clinical practice to distinguish between patients with receptor positive and negative tumours. However, from the clinical point of view, the most relevant border-line value should be that, above which patients will most probably respond to hormonal therapy and below which only a minor fraction of patients respond to that treatment and where other regimes should be considered. Jensen et al. (1976) have therefore chosen a higher cut-off point and the samples were thereby divided into ER rich (30% of the samples) and ER poor (70% of the samples). The response rate in the ER rich group, in their study, was 63% compared with 2.5% in the ER poor group. A similar indication of the importance of the quantification of ER is shown by McGuire (1980). By dividing the samples into four groups with different ER concentrations (<3, 3-10, 10-100 and >100 fmol/mg protein, respectively) a successively increased response rate (17%, 42%, 45% and 83%, respectively) was found.

ER and PgR are generally measured in tumour samples from breast cancer patients, for the clinical management of this disease. Steroid receptor determinations may also be of interest in other endocrine dependent malignant tumour disorders (e.g. endometrial carcinoma (Siiteri 1978, Kaupilla et al. 1982), ovarian carcinoma (Friedman et al. 1979, Holt et al. 1979), prostatic carcinoma (Ekman 1982, Wolf et al. 1985), leukemia and lymphatic malignancies (Rosen et al. 1983). Furthermore, steroid receptors have been found in a great number of other malignant disorders, which are more or less considered to be under endocrine control (Stedman et al. 1980), e.g. carcinoma of colon (Alford et al. 1979), kidney (Karr et al. 1983), pancreas (Greenway et al. 1981) and liver (Iqbal et al. 1983) as well as malignant melanoma (Neifeld and Lippman 1980) and meningioma (Martuza et al. 1981).

Breast cancer.

The hormone dependency of breast cancer was suggested as early as 1836, when Cooper in England observed fluctuations in tumour proliferation during different phases of the menstrual cycle (Cooper 1836). In 1896 Beatson (Edinburgh) removed ovaries from young women with advanced breast cancer and reported that some of the patients showed striking a regression of their metastatic lesions (Beatson 1896). When cortisol and other cortical hormones became available, adrenalectomy became another possible treatment for menopausal patients with advanced breast cancer (Huggins and Bergenstal 1952). The surgical excision of the pituitary gland became a further possible ablation of a hormone-influencing organ (Luft and Olivecrona 1953, Pearson et al. 1956). As an alternative for these surgical ablations, the administration of substances to alter the hormonal milieu, was found to be beneficial for a group of patients with breast cancer (Nathanson 1952). This endocrine therapy includes androgens (Goldenberg et al. 1973, Volk et al. 1974), large doses of estrogens (Haddow et al. 1944), progestins (Löber et al. 1981, Blossey et al. 1984), aminoglutethimide (Gale 1982, Harris et al. 1982) and estrogen antagonists such as tamoxifen (Cole et al. 1971), the latter being the most frequently used hormonal drug today (Mouridsen and Palshof 1978).

About one third of breast cancer patients with disseminated disease will respond to hormonal therapy. On the basis of clinical experiences, the patients most likely to benefit from hormonal therapy are those with a/ a long disease-free interval between mastectomy and the appearance of recurrences, b/ skin and lymph node metastases, c/ an earlier response to endocrine therapy. On the other hand very young women will less frequently respond to hormonal treatment (Henderson and Canellos 1980).

An attempt in the laboratory to predict the response to endocrine therapy, was made by Bulbrook and associates. They studied steroid hormone metabolites, especially from the adrenal and gonads, in the urine of breast cancer patients (Atkins et al. 1968). Although some relationship was found, the results were not sufficient to influence therapy decisions.

The rationale of steroid receptor determinations for the selection of patients for endocrine therapy was indicated when studying the uptake of tritiated hexaestrol, (Glascook and Hoekstra 1959) and estradiol (Jensen and Jacobson 1962), in estrogen responsive reproductive tissues (uterus, vagina and anterior pituitary) of laboratory animals. In 1961 Folca and co-workers found a correlation between the uptake of labelled hexaestrol in breast cancer tumours and the response of adrenalectomy in these patients (Folca et al. 1961). During the following decades extensive work has been performed in order to establish the role of estrogen receptor determination in predicting the response to hormone therapy in patients with advanced breast cancer disease (Jensen et al. 1975, McGuire 1978, Westerberg et al. 1978). PgR is claimed to be important also, since if present, it indicates an intact estrogen-ER mechanism (Horwitz and McGuire 1975, Horwitz 1981). Consequently, the predictive value of receptors has been improved by taking both ER and PgR into consideration. If both are positive, about 75-80% of the patients will respond to hormonal therapy, in contrast to less than 10% when receptors are lacking (McGuire 1980, Horwitz 1981).

There are several possible explanations for the lack of complete concordance between receptor status and the response to endocrine therapy (King 1975, Maass et al. 1980, Cowan and Lippman 1982). In this respect the assays may therefore be called "false negative" or "false positive".

"False negative" assays.

1. A "false negative" receptor measurement may be due to tumour tissue heterogeneity.
2. Since steroid receptors are thermolabile and unstable proteins, incorrect handling and storage of biopsies as well as methodological pitfalls may result in a "false negative" assay.
3. It may not be enough to measure ER and PgR in the samples. The interaction with other hormones (prolactin, glucocorticoids and androgens) and their receptors may also be of importance. (Allegra et al. 1979).
4. Receptors occupied by endogenous hormones, hormonal or anti-hormonal drugs are not measurable by the commonly used techniques and therefore a possible "false negative" assay might be obtained.
5. Due to the presence of endogenous or exogenous hormones, the receptor

may mainly be localized in the nucleus and therefore missed by the standard methods of today, which measure only cytoplasmic receptor (Garola and McGuire 1977).

The two latter points indicate that receptor values obtained in samples from premenopausal breast cancer patients, especially in periods of the menstrual cycle with high estradiol and progesterone levels, should be interpreted by caution (Nordenskjöld 1978, Saez et al. 1978).

6. Radiation treatment can produce artificially low ER values (Janssens et al. 1981).

7. In some few cases a change may occur in receptor content from negative in the primary tumour, in which the receptor content is measured, to positive in the metastases in which the receptor status is usually not determined (Harland et al. 1983).

8. The inadvertent assay of neighbouring non-malignant tissue due to omission of a pathological examination of the sample is another reason for "false negative" receptor values. Furthermore, it is important to obtain representative parts of the tumour including both peripheral and central parts.

"False positive" assays.

As mentioned above about 25-30 per cent of breast cancer patients with a ER and PgR positive sample do not respond to hormonal therapy.

1. The most common explanation is tumour heterogeneity (Seibert and Lippman 1982). Although a number of tumour cells may be receptor positive and thus making them measurable, a great proportion of the cells may be receptor negative and therefore refractory to hormonal therapy.

2. The interaction with other hormones and their receptors may also be of significance here. For example, it has been demonstrated that the effect of the progestins, megestrol acetate and medroxyprogesterone acetate, was not correlated with PgR but instead was significantly correlated with the amount of androgen and glucocorticoid receptors (Teuling et al. 1980).

3. A step distal to the initial hormone binding step may be non-functional. In the case of ER, such markers of a functional system are nuclear ER (Laing et al. 1977, Barnes et al. 1979), PgR (Horwitz 1981), peroxidase (Anderson et al. 1975, Duffy and Duffy 1977) and enhanced nucleoside incorporation (Lippman et al. 1976). As indicated above, at least the measurement of PgR has been shown to be of clinical significance (McGuire 1980, Horwitz 1981). The importance of measuring nuclear ER has also been reported (Leake et al. 1981, Cowan et al. 1984).

4. In the case of ER, it may also exist in a non-functional configuration (Wittliff et al. 1981) or the K_d -value may be too high (Braunsberg et al. 1975).

5. No current method gives information about all receptor criteria. The importance of this is indicated in the case of tyrosinase, present in

melanotic malignant melanomas, which mimics the estradiol binding to ER as measured with the DCC technique (McCarty et al. 1980). Other estradiol binding proteins, similar to tyrosinase, may exist in breast cancer samples.

6. The chosen cut-off point may be too low for distinguishing between positive and negative samples (Maass et al. 1980).

7. A change of receptor content from positive in the primary tumour to negative in the metastases may occur in some cases. Therefore, when possible, the receptor content should also be measured in the metastatic lesion (Harland et al. 1983, Gross et al. 1984).

8. Too strong criteria for objective response (Stoll 1977, Maass et al. 1980).

9. The patient will perhaps respond to another hormonal regimen (Bloom et al. 1983).

Besides their usefulness in the prediction of hormonal treatment of metastatic disease, receptor determinations have also been claimed to be of value for the prediction of the prognosis of the disease, in terms of a prolonged recurrence free survival (RFS). In several studies, a longer RFS has been demonstrated for breast cancer patients with receptor positive tumours when compared to those with receptor negative tumours (Knight et al. 1977, Hähnel et al. 1979, Godolphin et al. 1981). For the prediction of RFS, the measurement of PgR has been suggested to be of greater value than determination of ER (Clark et al. 1983). Several other investigators have, however, not been able to confirm the correlation between receptor status and RFS (Hilf et al. 1980 I, Shapiro et al. 1982, Howat et al. 1983). The initial prognostic significance of receptor determinations has in some studies also been found to disappear, when prolonging follow-up time (Hähnel et al. 1979, Aamdal et al. 1984). Other possible reasons for the contradictory findings will be further discussed (pages 47 and 48).

Patients with metastatic breast cancer and ER negative primary tumours have been suggested to have an increased probability of responding to cytotoxic chemotherapy (Allegria et al. 1978, Lippman et al. 1978). The opposite results, however, have been obtained by Kiang et al. (1978), Chang and Wergowske (1981), Mortimer et al. (1981). However, other studies have not been able to confirm, that the response to chemotherapy was correlated with receptor status (Hilf et al. 1980 II, Jonat et al. 1980).

Finally, some recent reports have demonstrated that adjuvant hormonal therapy was more efficient in prolonging RFS in breast cancer patients with receptor positive (or rich) tumour samples, compared to those with

receptor negative (or poor) tumours (Fisher et al. 1983, Ludwig Breast Cancer Study Group 1984, Wallgren et al. 1984, Rose et al. 1985, Stewart and Prescott 1985). However, in another trial, patients with ER negative and ER positive tumours responded equally well to adjuvant tamoxifen treatment with respect to RFS (Baum et al. 1985).

Endometrial carcinoma.

The uterine endometrium is another example of a hormone dependent tissue. Its growth and morphology are changing cyclically in response to estrogens and progestins. The normal endometrium has also been found to contain ER and PgR, and ER persists in most endometrial carcinomas. The ER concentration and the fraction of PgR positive samples have been found to be inversely correlated with the degree of histological differentiation of the tumour (Kauppila et al. 1982).

In 1961, Kelley, and co-workers reported that progestins could cause regression in about one-third of patients with metastatic endometrial cancer (Kelley and Baker 1961). Patients with more differentiated tumours showed a better response than those with poorly differentiated tumours (Malkasian et al. 1971). One possible explanation for the effect of progestins may be a reduced ER level and thereby an antiproliferative effect (Siiteri 1978).

From above is thus clearly indicated that ER and PgR assays may be of clinical importance in endometrial carcinoma, similar to that in breast cancer. To further investigate this importance, work is in progress at our, as well as other laboratories to further evaluate the measurements of ER and PgR in relation to the prognosis and the response to hormonal treatment in endometrial carcinoma.

ANTIESTROGEN BINDING SITES IN BREAST CANCER

Today, the antiestrogen tamoxifen (TAM) is the most common hormonal treatment in advanced breast cancer (Mouridsen and Palshof 1978). It is generally accepted that TAM exerts its antitumoural properties by blocking ER in the cytoplasm (Katzenellenbogen et al. 1979). Besides the blockade of ER, TAM may also act on the level of hypothalamus and pituitary by changing the concentration of circulating gonadotropins and prolactin and it has also been found to induce an increase of estradiol secretion in premenopausal breast cancer patients (Jordan 1982).

By using ³H-TAM, Sutherland et al. (1980 I and II) have been able to demonstrate an additional binding site for TAM, besides ER, in breast

cancer biopsy samples, namely the antiestrogen binding site (AEBS_{spec}). In breast cancer cell-lines the presence of AEBS_{spec} has been shown to be of importance for the effect of TAM on cell growth (Faye et al. 1983, Saez and Chouvet 1984). A possible role for AEBS_{spec} in breast cancer patients, treated with TAM, has not yet been evaluated.

ESTRAMUSTINE BINDING SITE IN BREAST CANCER

It is a well-known fact that the positive effects of cytotoxic treatment in cancer therapy must be evaluated against the often widespread and severe side effects. Therefore, based upon the action of steroids through their receptors in target cells, the idea of a directed therapy came up (Könyves and Liljekvist 1976). By adding nornitrogen mustard bound by a carbamate link to an estradiol molecule, it was thought that this substance (estramustine) should be specifically transported to ER containing tumours. The cytotoxic part of the molecule should then act locally. While estramustine phosphate was found to be effective in carcinoma of the prostate (Edsmyr et al. 1982), the initial clinical studies in breast cancer were not encouraging. In three investigations the response rate was 2/34, 17/44 and 2/16, which was considered too low for clinical use (Groupe Europeen du Cancer du Sein 1969, Alexander et al. 1979, Dawes 1982). Later studies have shown that estramustine does not bind to ER (Forsgren et al. 1979), but rather to a specific estramustine binding protein (EMBP), first found in the ventral prostate of rat (Forsgren et al. 1979, Björk et al. 1982). A binding site in breast cancer, similar to EMBP in the prostate, previously not reported, was studied in the present work.

AIMS OF THE STUDY

- To develop a method suitable for routine measurement of both estrogen and progesterone receptors in the same breast cancer biopsy sample and to investigate analytical steps critical for receptor recovery (I).
- To compare two techniques for estrogen and progesterone receptor determinations, dextran coated charcoal method with Scatchard analysis and isoelectric focusing (II).
- To compare and evaluate the choice of reference parameter (protein, DNA and wet weight) for the expression of steroid receptor content in breast cancer samples (III).
- To investigate the importance of steroid receptor determinations in samples from patients with breast cancer stage II for the prognosis of the disease (recurrence free survival, survival and survival after recurrence) in relation to the modality of adjuvant therapy (IV).
- To correlate estrogen and progesterone receptor contents in endometrial carcinoma with different clinical parameters, such as histological grade and thymidine incorporation (V).
- To provide further knowledge of the antiestrogen binding site in breast cancer as far as measurement, specificity, affinity to and relation to estrogen and progesterone receptors are concerned (VI).
- To investigate if there is a molecular basis, similar to the estramustine binding protein in prostatic carcinoma, to explain the effect of estramustine phosphate in some cases of breast cancer (VII).

MATERIAL AND METHODS

SAMPLING

Breast cancer. In all papers but number V, the principal tissue material was human breast cancer biopsies. These samples were obtained at operation from 15 hospitals (Southern Swedish Health Care Region), were freed from fat, blood clots etc., and transported in solid CO₂ or fresh on ice to the receptor laboratory at the Department of Oncology, University Hospital, Lund. On the arrival at the laboratory the material was minced, frozen and kept in liquid nitrogen for not more than two weeks until analysis.

For control experiments excessive tissue samples were frozen and stored at -70°C.

Endometrial carcinoma. Curettage material was obtained from patients referred for primary treatment to the Gynecological Section, Department of Oncology, Lund. Part of the material was fixed in neutral formalin and further processed for histopathology. Within 30 minutes the remaining part was frozen in liquid nitrogen and stored for not more than two weeks until analysis (V).

For control experiments (I,VI) excessive tissue was frozen and stored at -70°C.

Normal tissues. Normal uterine tissue (rat and rabbit) was used for control experiments (I,II,VI). It was frozen and stored in liquid nitrogen or at -70°C.

CHEMICALS AND LIGANDS

Chemicals. All chemicals in the present investigations were of analytical grade and are described in detail in paper number I.

Ligands. The radioactive ligands (³H-estradiol (I-VII), ³H-R 5020 (I-VII), ³H-tamoxifen (VI), ³H-hydroxytamoxifen (VI) and ³H-estramustine (VII) as well as the non-radioactive ligands are described in detail in respective paper.

PREPARATION OF SUPERNATANTS

These analytical steps are described in detail in paper I and can be summarized according to the following brief description:

The specimens were weighed and homogenized in TEM buffer (Tris-HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM; pH 7.4 (sometimes with the addition of 1.0 mM NaN₃)) with an all-glass Potter-Elvehjem homogenizer at 0°C. After homogenization, volume samples were removed for DNA determination (see page 29), whereafter the remaining part was centrifuged at 20,000xg for 10 minutes. The supernatant was used for the following analysis.

1. ER-assay (I-VII).
2. PgR-assay (I-VII).
3. Analysis of antiestrogen binding site (VI).
4. Analysis of estramustine binding site (VII).
5. Protein estimation (ODU₂₈₀₋₃₁₀; I-VII).
6. Protein determination according to Lowry et al. [(1951); I, III-VII].

ER AND PgR ASSAY

These assays are described in detail in paper I. ER content in the supernatant was measured with isoelectric focusing in polyacrylamide gel (IF). Presence of ER was indicated by a radioactive peak at pH 6.3-6.5. The specificity and saturability of this peak was sometimes controlled by the addition of a 100-fold excess of non-radioactive estradiol.

The concentration of PgR was assessed with the multiple point dextran coated charcoal (DCC) technique and Scatchard analysis.

For comparison, in paper II, ER was also measured with the DCC technique and Scatchard analysis, as well as IF, was used also for PgR assay.

Methodological modifications.

In May 1980 modifications of the analytical procedures were performed resulting in an increased sensitivity and recovery of the measurable amounts of ER and PgR. These modifications are in detail described in paper I and are summarized in Tables 1 and 2.

In order to correlate clinical results and receptor values (as in paper IV) it was necessary to adjust values obtained prior to the methodological modifications. For this reason control experiments, using breast cancer samples, were performed.

A comparison of the two methodological variants in each sample showed an increase of the measurable amount of ER by a mean factor of 2.0 after the modifications (n=24). Similar control experiments were performed for PgR. The mean calculated conversion factor for PgR was 1.5 (n=20).

In the present study, these conversion factors were applied only in paper IV, in which the prognostic significance of ER and PgR in stage II breast cancer were investigated. In this paper the receptor measurements were performed in order to distinguish between patients with receptor positive and receptor negative tumour samples. The validity of the above mentioned conversion factors was therefore tested by dividing the samples into two groups, one receptor positive and one receptor negative. The test of the validity was performed according to the following:

Table 1.
Analytical steps subject to modification in the
ER assay (isoelectric focusing).

Analytical step	Methodological variant	
	before "May 1980"	after "May 1980"
3Addition of H-estradiol	prior to analysis	after homogenate centrifugation
Mercapto- ethanol	absent	present
DCC ¹ incuba- tion time	10 min.	1 min.
Charcoal conc. (w/v)	1.0%	0.3%
Protein determination	before DCC treatment	after DCC treatment

¹dextran-coated charcoal

Table 2.
Analytical steps subject to modification in the
PgR assay (dextran-coated charcoal method with
Scatchard analysis).

Analytical step	Methodological variant	
	before "May 1980"	after "May 1980"
Homogenate centrifugation	100,000xg 60 min.	20,000xg 10 min.
Addition of glycerol	prior to analysis	after homogenate centrifugation
Sulphydryl- compound	dithio- threitol	mercapto- ethanol
DCC ¹ incuba- tion time	30 min.	10 min.
Protein determination	before DCC treatment	after DCC treatment

¹dextran-coated charcoal

1/ It seems reasonable to assume that the distribution of ER and PgR should be about the same before and after the methodological modifications in May 1980. In order to test this assumption a material from patients with breast cancer stage II was applied. As, at least ER, is dependent on the menopausal status, the comparison of receptor distributions was performed separately for samples from pre- and post-menopausal patients. Tables 3 and 4 demonstrate a good agreement of the receptor distribution for the two methodological variants, with the exception of PgR for premenopausal patients. This divergent finding may, at least partly, be explained by the smaller amount of samples in this group. The distributions of PgR, for the two variants, were therefore also studied in a larger material of patients with primary breast cancer. No compensation for menopausal status or stage of disease was made. The fraction of PgR positive samples was then 74/164 (45%) with the first variant and 358/808 (44%) with the last variant.

Table 3.
ER distributions and mean ER concentrations
(ER) for two methodological variants in respect
to menopausal status for patients with breast
cancer stage II.

Method variant	Premenopausal				Postmenopausal			
	ER+ %	ER- %	ER ¹ %	n ²	ER+ %	ER- %	ER ¹ %	n
before "May 1980" ³	56	44	19	55	74	26	61	114
after "May 1980" ⁴	55	45	38	135	73	27	134	191

¹fmol/mg protein

²number of patients

³cut-off point 5 fmol/mg protein

⁴cut-off point 10 fmol/mg protein

Table 4.
PgR distributions and mean PgR concentrations
(PgR) for two methodological variants in
respect to menopausal status for patients with
breast cancer stage II.

Method variant	Premenopausal				Postmenopausal			
	PgR+ %	PgR- %	PgR ¹ %	n ²	PgR+ %	PgR- %	PgR ¹ %	n
before "May 1980" ³	63	37	160	41	42	58	92	84
after "May 1980" ⁴	48	52	200	139	44	56	150	193

¹fmol/mg protein

²number of patients

³cut-off point 20 fmol/mg protein

⁴cut-off point 30 fmol/mg protein

2/ The conversions performed in the present study should have their greatest importance around the cut-off points (10 fmol ER/mg protein and 30 fmol PgR/mg protein). In five of the 24 control experiments, the ER concentrations were 11, 0.51, 12, 3.7 and 3.4 fmol/mg protein, respectively, with the first variant. The corresponding figures of these samples, when measured after the modification of the method, were: 20, 2.0, 23, 6.3 and 6.9, respectively.

This indicates that the conversion factor was also valid for samples with ER concentrations around the cut-off point. For PgR, the number of control experiments with concentrations around the cut-off point was too small to allow the same comparison.

3/ The use of the conversion factors (2.0 (ER) and 1.5 (PgR)) implies that samples with an ER concentration between 5 and 10 fmol/mg protein, or a PgR concentration between 20 and 30 fmol/mg protein, as obtained with the first methodological variant, are changed from negative to positive. In paper IV, this applies only for 14 (2.6%, ER) and 6 (1.1%, PgR) of 539 samples.

4/ The results in paper IV, where receptor values from both methodological variants were included, were furthermore compared with those results obtained when only values from the last variant were included. No noteworthy differences were thereby obtained regarding ER and PgR as prognostic indicators (recurrence free survival and overall survival).

Taken together, these four points strongly suggest that no influence on the results has been caused by the introduction of the new methodological variant (May 1980), with the concomitant use of conversion factors for comparison with older results.

ANTIESTROGEN BINDING SITE AEBS_{spec} (VI)

AEBS_{spec} was measured with the DCC technique and Scatchard analysis. To study its specificity and ligand affinity to AEBS_{spec}, supernatant samples were incubated with different ³H-ligands at one concentration, together with increasing concentrations (0-50 μM) of different non-radioactive ligands.

ESTRAMUSTINE BINDING SITE (EMBS) (VII)

Analysis of EMBS was performed with the IF technique in polyacrylamide gel, using ³H-estramustine as the radioactive ligand. Compared with the corresponding ER assay, here the radioactivity was measured over a wider pH-range (3.5-9.5) and no trypsin and CaCl₂ were added.

PROTEIN AND DNA DETERMINATION

Protein determination was performed according to Lowry et al. (1951) with lyophilized bovine serum albumine as the standard.

The protein content was, furthermore, estimated by measuring the difference in absorbance at 280 nm and 310 nm (ODU₂₈₀₋₃₁₀; ODU = optical density unit). One ODU₂₈₀₋₃₁₀ has been found to correspond to 0.7 mg of protein (Wrange et al. 1978). In the ER assay with IF, 2.5 μg of trypsin was added per ODU₂₈₀₋₃₁₀ in order to partially digest the ER-complex from 8 S to 4 S (Wrange et al. 1978).

DNA content was measured with the diphenylamine reaction described by Burton (1956) with calf thymus as the standard.

STATISTICAL METHODS

The comparison of differences were performed with chi-square (χ^2) analysis (III, IV, VII), Spearman's correlation (III, IV, V, VI, VII), Fisher's exact test (V), Mann-Whitney's test (III) and by generalized Wilcoxon test (IV).

RESULTS

IMPROVED METHOD FOR ASSAY OF ESTRADIOL AND PROGESTERONE RECEPTORS WITH SPECIAL REFERENCE TO BREAST CANCER (I).

In order to make it possible to measure both estradiol (ER) and progesterone receptors (PgR) in the same 20,000xg supernatant sample from breast cancer biopsies slight modifications of previously used analytical procedures were carried out. The modifications concern mainly buffer contents and homogenate centrifugation conditions. In the ER assay mercaptoethanol was added to the buffer whereas ^3H -estradiol was added after centrifugation instead of prior to analysis. For the assay of PgR dithiothreitol was exchanged for mercaptoethanol and glycerol was added after homogenate centrifugation instead of prior to analysis. These modifications were in control experiments shown not to have any noteworthy influence on receptor recovery.

A comparison between two g-values (20,000xg and 100,000xg) was performed because the use of 20,000xg instead of 100,000xg would avoid the costly purchase of an ultracentrifuge. At the lower g-value a mean increase of PgR recovery of 19 per cent was obtained. The corresponding increase for ER was 34 per cent. As will be discussed later (page 42), this difference was considered to be of no noteworthy importance for the clinical evaluation of receptor content in breast cancer.

After the initial analytical steps, common for ER and PgR, ER was assayed with isoelectric focusing (IF), whereas PgR was measured with the dextran coated charcoal (DCC) method and Scatchard analysis. In both techniques, the separation of specifically from non-specifically bound ^3H -ligand was performed with DCC. A lower charcoal concentration (0.3% instead of 1.0% (w/v), final concentrations) and a shorter incubation time (1 minute instead of 10 minutes) was found to give a considerable increase of ER recovery. The incubation time was found to be more important for ER recovery than the charcoal concentration. As far as PgR assay is concerned, the incubation time was reduced from 30 to 10 minutes. The charcoal concentration here was unchanged (0.3% (w/v)).

Isoelectric focusing of ER makes it possible to measure 1.0 fmol specifically bound ^3H -estradiol per ml 20,000xg supernatant. With the above mentioned modifications, ER was detected in 95 % (previously 75 %) of the samples and the median ER concentration increased from 0.6 fmol/mg wet weight (n=607) to 1.5 (n=434). The frequency of PgR positivity was 59 % (previously 64 %) with an increase in the median PgR value of 30 % with

the present modification (n=355), compared with the previously used method (n=339).

The level of sensitivity for PgR assay was 5-10 fmol specifically bound ³H-R 5020 per ml 20,000xg supernatant.

A COMPARISON OF TWO STEROID RECEPTOR ASSAYS IN BREAST CANCER: DEXTRAN COATED CHARCOAL AND ISOELECTRIC FOCUSING (II).

Isoelectric focusing (IF) was compared with the dextran coated charcoal assay (DCC) and Scatchard analysis. As far as the ER assays are concerned, a comparison of 46 samples gave a very good concordance ($r_s=0.92$) between the two techniques. The ER recovery was, however, found to increase twice (median value, 42 samples positive with both techniques) when using DCC with Scatchard analysis instead of IF. The highest relative increase was found at the lowest ER levels.

A similar comparison of PgR recovery in 43 samples also showed a very good correlation between DCC with Scatchard analysis and IF ($r_s=0.91$). The difference in recovery was even more pronounced than for ER assay; the recovery increased by a median factor of 3.9 in 34 samples, positive with both techniques.

In order to study the reproducibility of the respective method, 4-9 samples from the same rat or rabbit uterus, 20,000xg supernatant, were analyzed separately. This was done at different levels of receptor concentration. With the exception for the lowest ER concentration as measured with IF, the deviation from the mean value was generally less than 10 %. This was true for both IF and DCC with Scatchard analysis. As far as PgR was concerned, the variations were somewhat higher than for the corresponding ER study.

OBSERVATIONS ON WET WEIGHT, PROTEIN AND DNA AS REFERENCE FOR STEROID RECEPTORS IN MALIGNANT MAMMARY TUMOURS (III).

DNA, protein and wet weight were compared as reference parameters for expression of ER and PgR concentrations in 235 breast cancer samples. 216 (92%) of these samples had ER concentrations above the level of detection (1.0 pM), whereas 102 (47%) of the 217 samples evaluable for PgR had a PgR concentration above the level of detection (5-10 pM). In this paper, receptor positivity was defined as concentration values above the level of sensitivity for ER and PgR, respectively.

The quotient between the highest and lowest ER value (on wet weight basis) was approximately 7,200. For PgR the corresponding quotient was 280. The ratio between the highest and lowest DNA and protein values (per mg wet weight) were 61 and 13, respectively.

Correlations of receptor concentrations expressed per unit wet weight, protein and DNA were performed and showed an overall strong positive correlation ($r_s > 0.91$), irrespective of reference parameter. The choice of parameter could, however, be of importance in single cases. When arbitrarily chosen cut-off points were used, a few samples in this material switched from below to above the border-line value or vice versa depending on the choice of parameter.

In the present material ER was not detected in 19 samples. Ten (34%) of these samples were among the 29 with the lowest DNA content, which in this context was defined as below one standard deviation (SD) from the mean DNA value. The expected percentage, if ER and DNA were independent, would be 8.1 ($p < 0.001$). When comparing the mean DNA content for the 19 ER negative samples with the 216 ER positive ones a significantly ($p < 0.005$) lower mean DNA content (per mg wet weight) was found among the ER negatives, compared with the ER positives (2.3 ± 2.3 mg/g and 3.4 ± 1.9 mg/g, respectively). In a corresponding way, ER rich (the highest ER positive quartile) samples ($n=54$), were overrepresented among those with a high DNA content. The ER rich biopsies also had a significantly ($p < 0.0001$) higher mean DNA content compared with samples with lower ER concentrations ($n=181$; 4.1 ± 1.6 mg/g and 3.1 ± 2.0 mg/g, respectively).

As far as PgR was concerned, no difference in mean DNA content was found between PgR negative ($n=115$) and PgR positive ($n=112$) samples. In agreement with ER, PgR negative biopsies were overrepresented in a group of tumours with DNA content below one SD from the mean DNA value ($n=27$). Also in agreement with the corresponding ER study, PgR rich biopsies (the highest one third of PgR positives; $n=34$) had a significantly ($p < 0.05$) higher mean DNA content per mg wet weight compared with the remaining 183 samples (4.0 ± 2.2 mg/g and 3.2 ± 1.9 mg/g, respectively). However, in contrast to ER, PgR rich samples were not found to be overrepresented in samples with DNA content higher than one SD from the mean value.

Furthermore, it was found that ER and PgR negative tumours were overrepresented in the group of samples with a low DNA concentration in the homogenate (< 36 μ g DNA/ml homogenate; i.e. mean - 1 SD). Five (42%) of 12 samples in this group were ER negative, whereas 10 (91%) of 11 samples were PgR negative. The expected percentages were 8.1 ($p < 0.001$) and 53 ($p < 0.05$), respectively.

A similar investigation was also performed for protein content (per mg wet weight). Subgroups of tumours characterized by low or high protein contents in combination with low or high ER or PgR concentrations could not be distinguished.

PROGNOSTIC SIGNIFICANCE OF ESTROGEN AND PROGESTERONE RECEPTORS IN BREAST CANCER STAGE II (IV).

Patients.

Steroid receptor assays were made in biopsies obtained from patients participating in a prospective controlled clinical study on adjuvant therapy of stage II breast cancer, organized by the South Swedish Breast Cancer Group. The total number of randomized patients in the trial was 1123. The trial started in January 1978 and was closed in November 1983 for premenopausal patients and in December 1984 for postmenopausal patients. The present data were evaluated in March 1985, at which time the median observation time was 39 (range 14-85 months) for premenopausal and 32 (range 2-86 months) for postmenopausal patients.

Premenopausal patients were randomized to one of three postoperative treatment groups: radiotherapy alone, radiotherapy and cyclophosphamide, and cyclophosphamide alone.

Postmenopausal patients were randomized to one of the following postoperative treatment groups: radiotherapy alone, radiotherapy and tamoxifen (TAM), and TAM alone. More details concerning patients, treatment and follow-up are described in paper IV.

Sampling of tumour material.

During the first five and a half years of study 162 tumour samples were receptor determined at another hospital. Of the remaining 961 samples, 801 (83.4%) were sent to our laboratory. Fifty-nine (7.4%) of these 801 samples arrived at an unsuitable temperature, 28 (3.5%) were considered too small for a reliable analysis, and 22 (2.7%) samples were taken from patients under endocrine treatment including contraceptives. Adequate determination of ER and/or PgR could thus be performed in 692 (86.4%) of the 801 samples, but in the present follow-up study only samples from 539 patients assayed for both ER and PgR were included.

The patients with ER and PgR determined samples (n=539) and those without both receptor measurements (n=584) were equal with regard to age, lymph-node involvement and distribution between different treatment groups. Tumours greater than 2 cm in diameter were, however, more often analyzed

for receptors than tumours less than 2 cm (54% versus 37%).

Receptor distributions.

ER concentrations ≥ 10 fmol/mg protein was found in 104 of 184 (57%) premenopausal patients and in 243 of 355 (68%) postmenopausal patients. For PgR ≥ 30 fmol/mg protein, the corresponding figures were 92 of 184 (50%) and 144 of 355 (41%), respectively. When taking both ER and PgR status into consideration the distributions of those patients without both receptors (ER-PgR-) and those with both receptors (ER+PgR+) were almost the same for pre- and postmenopausal patients (35% versus 30% and 42% versus 39%, respectively). Patients with ER+PgR- tumours were, however, more often found in the postmenopausal group (15% versus 29%), whereas the opposite was found for ER-PgR+ patients (8% versus 1%).

The proportion of ER+ and PgR+ samples were similar, whether axillary nodes were involved or not.

Follow-up results for premenopausal patients.

Recurrence free survival (RFS). At the routinely chosen cut-off points (10 fmol ER/mg protein and 30 fmol PgR/mg protein), RFS was not found to be related to receptor status for the tumour, irrespective of randomization into one of three subgroups receiving different postoperative therapy. However, by changing the cut-off points to 40 or 50 fmol ER/mg protein and ≥ 40 fmol PgR/mg protein a significantly longer RFS was found for patients with receptor values above the cut-off point in the tumour compared with those patients with receptor values below the cut-off point in the tumour. The p-values at the cut-off point (50 fmol/mg protein) with the strongest statistical significance were 0.026 (ER) and 0.0087 (PgR).

Survival. Statistically significant differences were observed in favour of patients with receptor positive tumours (ER+ (≥ 10 fmol/mg protein), PgR+ (≥ 30 fmol/mg protein) and ER+PgR+) when compared to patients with receptor negative tumours (ER-, PgR- and ER-PgR-; $p=0.009$, $p=0.007$ and $p=0.003$, respectively).

Survival after recurrence was significantly longer in ER+ patients than in ER- patients ($p=0.006$).

Follow-up results for postmenopausal patients.

RFS. No difference was found between receptor positive and receptor negative patients, not treated with adjuvant TAM. In contrast to premenopausal patients, this finding was not influenced by the chosen cut-off point. However, patients with receptor positive tumours, treated with adjuvant TAM, with or without postoperative radiotherapy had a significant longer RFS compared with the corresponding group of patients with

receptor negative tumours (ER+ versus ER-, $p=0.0001$; PgR+ versus PgR-, $p<0.0001$). This difference was not more pronounced when both ER and PgR status was taken into consideration, but patients with ER+/PgR+ tumours had a significantly better RFS compared with those patients with ER+/PgR- tumours ($p=0.004$).

Survival. Patients with ER positive tumours, not treated with adjuvant TAM, had a better survival rate than corresponding patients with ER negative tumours ($p=0.04$). This difference was not found between PgR positive and PgR negative patients. For the group of patients treated with adjuvant TAM, the difference in survival between patients with ER positive and ER negative tumours was more pronounced ($p=0.004$) than for patients not treated with adjuvant TAM, and this was also true for patients with PgR positive tumours compared with patients with PgR negative tumours ($p=0.002$).

No difference in survival after relapse, according to receptor status was observed in this patient group, treated with adjuvant TAM.

PLASMA STEROID HORMONES, CYTOSOL RECEPTORS AND THYMIDINE INCORPORATION RATE IN ENDOMETRIAL CARCINOMA (V).

Curettage material from 92 endometrial carcinomas was obtained in connection with primary treatment. All were ER positive and 88% were PgR positive. The fraction of PgR positive samples and mean ER and PgR concentrations, expressed as fmol/mg protein or DNA were strongly correlated with the degree of tumour differentiation. Thus, well differentiated (WD) tumours had a higher fraction of PgR positive cases as well as higher mean ER and PgR concentration when compared with the poorly differentiated (PD) ones. For the moderately differentiated (MD) tumours an intermediate appearance was found. All the WD tumours contained high concentrations of ER. For both MD and PD tumours, two subgroups could be distinguished, one with high ER concentrations, corresponding to WD, and one with low ER concentration levels. The subgroup with low ER concentrations was larger for PD tumours compared with MD tumours. The PgR findings were about the same as those for ER.

Plasma estradiol concentration was positively correlated with tumour differentiation ($p<0.05$), ER ($p<0.05$) and PgR ($p<0.001$), whereas a negative correlation ($p<0.04$) was found between plasma estradiol and rate of DNA synthesis, as represented by thymidine incorporation in tumour cells. The latter also showed a negative correlation ($p<0.04$) with PgR. Plasma progesterone concentrations did not correlate with any of the variables studied.

ANTIESTROGEN BINDING SITES IN HUMAN BREAST CANCER BIOPSIES. MEASUREMENT, LIGAND SPECIFICITY AND -AFFINITY, AND CORRELATION TO ESTROGEN AND PROGESTERONE RECEPTORS (VI).

Specific binding sites for the antiestrogen tamoxifen (AEBS_{tot}) as measured with the dextran coated charcoal technique and Scatchard analysis were found in 43 of 44 breast cancer biopsy samples. AEBS_{tot} has in the present study been considered to consist of ER and another binding site ($\text{AEBS}_{\text{spec}}$) for the antiestrogen tamoxifen (TAM) and its main metabolites (4-OH-TAM and N-d-Me-TAM).

The mean concentration of AEBS_{tot} ($1,000 \pm 600$ fmol/mg protein) was higher than those of ER and PgR (100 ± 180 and 240 ± 580 fmol/mg protein, respectively). The dissociation constant (K_d) was found to lie between 0.26 to 7.7 nM (2.1 ± 1.8), indicating that $\text{AEBS}_{\text{spec}}$, as well as steroid receptors, is a high-affinity binding site. When comparing the content of AEBS_{tot} and steroid receptors, a weak positive correlation was found between AEBS_{tot} and ER ($p=0.041$), whereas no correlation between AEBS_{tot} and PgR could be demonstrated.

The study of the relative binding affinity (RBA; for definition see paper VI) of estradiol and antiestrogens to ER and $\text{AEBS}_{\text{spec}}$, respectively, showed that compared with estradiol (100%), 4-OH-TAM was found to have a higher affinity to ER (47%) than had TAM (0.8%) and N-d-Me-TAM (0.7%).

The RBA of estradiol, TAM and its main metabolites to $\text{AEBS}_{\text{spec}}$ was studied in ER negative samples (e.g. below the level of detection (1.0 pM)). Any binding by estradiol to $\text{AEBS}_{\text{spec}}$ could not be demonstrated. After two hours incubation, the RBA of TAM, 4-OH-TAM and N-d-Me-TAM was 100%, 16% and 9.3%, respectively, with ^3H -TAM as the radioactive ligand and 100%, 67% and 25%, respectively, with ^3H -4-OH-TAM as the ligand. When prolonging the incubation time to 20 hours the difference in RBA between TAM and 4-OH-TAM with ^3H -TAM as the ligand disappeared.

In order to get the concentration of $\text{AEBS}_{\text{spec}}$ the content of ER should be subtracting from that of AEBS_{tot} . As estradiol binds to ER but not to $\text{AEBS}_{\text{spec}}$ it is reasonable to assume that by adding excess estradiol, ER will be blocked. When incubating with ^3H -TAM after such ER blockade, only $\text{AEBS}_{\text{spec}}$ would then be measured. To test this hypothesis, the inhibition of excess non-radioactive estradiol on ^3H -TAM binding was compared with ER content, as measured with IF in the same sample. A correlation was however only found in those samples (6/43) with a comparatively high ER content (e.g. $\text{ER}/\text{AEBS}_{\text{tot}} \geq 0.20$). Consequently, in a great proportion of the samples (37/43) the inhibition of excess estradiol on ^3H -TAM binding showed no correlation with ER content. Therefore, this does not seem to

be a suitable method for measuring the concentration of $\text{AEBS}_{\text{spec}}$. However, when using ^3H -4-OH-TAM instead of ^3H -TAM as the radioactive tracer, the correlation between the inhibition of excess of non-radioactive estradiol on ^3H -4-OH-TAM binding was better correlated with ER content, even at low ER levels. At very low ER concentrations no correlation was found. The influence of this non-concordance, at low ER levels, on the measurable amount of $\text{AEBS}_{\text{spec}}$ was, however, considered to be of minor importance.

By subtracting estradiol inhibition from the total ^3H -4-OH-TAM binding (AEBS_{tot}), the concentration of $\text{AEBS}_{\text{spec}}$ was calculated in 28 samples. The mean concentration of $\text{AEBS}_{\text{spec}}$ was found to be 860 ± 590 fmol/mg protein, which should be compared with 120 ± 170 and 190 ± 330 , being the corresponding ER and PgR concentration values. In agreement with AEBS_{tot} , $\text{AEBS}_{\text{spec}}$ showed a weak positive correlation with ER, no correlation with PgR and showed also similar K_d -values (2.7 ± 1.7 nM).

ESTRAMUSTINE BINDING SITE IN HUMAN BREAST CANCER BIOPSY SAMPLES - ITS RELATION TO ESTROGEN AND PROGESTERONE RECEPTOR LEVELS, AGE AND MENOPAUSAL STATUS (VII).

The binding of ^3H -estramustine was studied in 306 breast cancer biopsy samples with IF. In 74 of these (24%) an estramustine binding site (EMBS) was demonstrated at pH 4.8-4.9. Presence of EMBS was significantly ($p < 0.001$) correlated with negative or low ER and PgR values (< 10 fmol ER and < 30 fmol PgR/mg protein, respectively). EMBS positive samples have lower mean ER and PgR concentration values (20 and 57 fmol/mg protein, respectively) compared with EMBS negatives (160 and 200 fmol/mg protein, respectively).

When dividing the samples into two groups depending on menopausal status, EMBS positivity was found in 31% (26/83) of the samples from pre- and peri-menopausal patients and in 22% (48/223) of the samples from postmenopausal (e.g. more than five years after the last regular menstrual bleeding), respectively.

The age distribution of patients with respect to EMBS status was also studied. Pre- and peri-menopausal patients younger than 50 years showed no difference in the fraction of EMBS positive (26%) cases between different decades. Pre- and peri-menopausal patients older than 50 years ($n=22$) were, however, more often EMBS+ (45%). For postmenopausal patients the highest EMBS+ frequency was found between 50 and 59 years of age

(41%). With increasing age the percentage of EMBS+ was then successively falling. Only 3 (7.5%) of 40 samples from patients older than 82 years were EMBS+.

With regard to ER and PgR status, the opposite pattern was obtained. Samples from older postmenopausal patients were more often receptor positive than those from younger postmenopausals. For pre- and peri-menopausal patients younger than 50 years of age, only slight differences in receptor status between age groups could be demonstrated.

When examining the correlation between EMBS and receptor status in relation to menopausal status, it was found that a statistically significant correlation between EMBS+ and low or negative receptor concentrations was restricted to samples from postmenopausal patients.

DISCUSSION

The use of steroid receptor determinations in the clinical management of breast cancer has stressed the importance of improvement and standardization of available analytical assays. The reason for this is the lack of complete concordance between the results of steroid receptor assays and the clinical response to hormonal treatment. As breast cancer is a multifactorial disease, it is of course reasonable to assume that other factors than steroid receptors may be of importance for response to endocrine therapy. But still, the handling of tissue samples for receptor determination, as well as the performance of the analytical assay, have been considered by different investigators to be of great importance for the reliability of steroid receptor measurements (DeSombre et al. 1979).

METHODOLOGICAL ASPECTS

The present series of investigations were originally started at our laboratory with the aim to establish a standard measurement for both ER and PgR in the same breast cancer sample. Methods for the determination of both receptors in the same sample should have several advantages, since a/ smaller amount of tissue should be needed, b/ the receptor profile should be more representative for the biopsy material under analysis, c/ the handling of the samples should be more uniform, d/ the assay should be less time consuming, and e/ both ER and PgR should be measured in a greater proportion of samples.

By modifying previously used techniques, it became possible to combine the isoelectric focusing of ER with the multiple point dextran-coated charcoal assay and Scatchard analysis for PgR in the same sample (I). Certain analytical steps were then found to be of great importance for receptor recovery.

The g-value for the homogenate centrifugation was shown to influence the recovery of both ER and PgR. A lower g-value (20,000xg) was found to increase ER recovery by a mean value of 34% as compared with a higher g-value (100,000xg; n=20; human breast cancer, rat and rabbit uterine tissue). For PgR the corresponding mean increase was 19% (n=21). These results are in agreement with other studies (Hawkins et al. 1975, van Netten et al. 1977) and may support the findings by Parikh et al. (1980), who in the microsomal fraction found binding sites for estrogens, with receptorlike properties. To study whether the choice of g-value is of significant importance for the evaluation of the receptor content,

further samples have been measured. When using Spearman's rank-correlation for comparing ER recovery at 20,000xg with that at 100,000xg, a r_s -value of 0.98 ($p < 0.001$, $n = 26$) was obtained, which indicates that the difference between g-values (20,000xg and 100,000xg) is of no noteworthy importance for the clinical evaluation of receptor content in breast cancer. The corresponding figures for PgR were 0.99 (r_s ; $p < 0.001$; $n = 26$).

Treatment with dextran-coated charcoal (DCC) is another step, which has been shown to influence the measurable amount of receptors. This treatment is used in both the IF method and DCC method with Scatchard analysis, in order to remove non-specifically bound hormone from receptor-bound (I).

During the isoelectric focusing procedure, used for ER assay in the present work, the receptor-bound and non-specifically bound ^3H -estradiol are separated due to different charge properties in the pH-gradient. As DCC treatment has been shown to reduce ER recovery, it is therefore possible to use a milder incubation with DCC when using the IF method. For ER assay with IF, it has in the present work been shown that an incubation time of 1 minute and a charcoal concentration of 0.3% (w/v, final concentration) was sufficient to remove adequate amounts of excess ligand, so that remaining non-specifically bound ^3H -estradiol did not significantly interfere with the analysis. The incubation time (1 minute compared to 10 minutes) was more important than the charcoal concentration (0.3% compared to 1.0%) for ER recovery. The mean increase of ER recovery with the shorter charcoal incubation time was 37% ($n = 14$), whereas the mean increase with the lower charcoal concentration was 7.1% ($n = 14$). When ER was measured with IF, without any DCC treatment, the background activity between the application point and the isoelectric point of ER increased, which resulted in less distinct chromatographs.

For PgR assay with DCC method and Scatchard analysis, a longer incubation time (10 minutes) with DCC was necessary to remove enough unbound and non-specifically bound ligand in order to get distinct analytical curves. These experiments, together with those of others (Ralet and Brombacher 1981), have pointed out that the incubation with DCC is a critical step for receptor measurement. It is therefore desirable that DCC treatment should be performed under as standardized conditions as possible, including charcoal concentration, incubation time and protein content. Especially at a low protein concentration, it has been demonstrated that an underestimation of ER content occurs, presumably due to adsorption of ER to the charcoal. (Powell et al. 1981).

Besides g-value at the homogenate centrifugation and DCC treatment,

several other analytical steps have also been considered to be of importance for the reliability of receptor assays. Some of these further analytical steps have been evaluated at our laboratory (unpublished data).

Sampling of tumour material. The importance of a correct handling and storage of breast cancer tissues has been discussed in Introduction (page 15).

As our laboratory gets samples from 15 different hospitals (the transportation distance being up to 200 km) it is of great importance to establish correct routines for tissue handling at the hospitals. The recommendations from our laboratory is that tissue material after operation should be divided into two parts - one for histopathological examination and one for receptor analysis. The latter piece should immediately be kept on ice and within one hour frozen at -70°C . After transport to the receptor laboratory in solid CO_2 or fresh on ice, the samples are kept in liquid nitrogen for not more than two weeks until analysis. Initial transportation problems have successively been reduced. During the first year (1978), 11% (31/292) of the samples arrived at the laboratory thawed, which should be compared with 1.2% (8/686) being the corresponding figure during 1984. To examine possible differences between hospitals, the distribution of receptor status (positive and negative) and mean values has been studied, without finding any major difference between the 15 hospitals. It seems, therefore, as if the handling of the tissue prior to its arrival to the laboratory has not influenced the results in the present study.

The preparation of the homogenate involves choice of buffer, protein concentration and homogenization technique. Tris-EDTA buffer at pH 7.4 is one of the most commonly used. The use of mercaptoethanol instead of dithiothreitol was in control experiments shown not to influence PgR values. As far as the protein concentration is concerned, it is recommended to be above 1 mg/ml (Seibert and Lippman 1982). At our laboratory, a protein concentration down to 0.5 mg/ml was considered to give reliable results, because 1/ the protein content was measured after DCC treatment, which reduces the protein concentration by a mean value of 28% ($\text{SD}=31\%$; $n=31$) and 2/ in control experiments it has been shown that receptor content was not influenced by a protein concentration down to 0.7 mg/ml prior to DCC treatment. Other studies have also demonstrated that a protein concentration of 0.7 mg/ml is sufficient to get adequate receptor measurements, at least when bovine serum albumin was added (Powell et al. 1981). At lower protein values the receptor recovery was, in agreement with other investigations, found to be reduced.

The importance of the choice of homogenization technique has not been evaluated at our laboratory. The use of a microdismembrator, which pulverizes the tissue chilled in liquid nitrogen, is the most commonly used and recommended technique of today. At our laboratory, this analytical step is performed with a glass-glass homogenizator.

Aspects on the homogenate centrifugation have already been discussed above.

Incubation with ^3H -ligand. After the homogenate centrifugation, the supernatant is incubated with radioactive ligand. Again, a great number of possibilities exist including choice and concentration of ^3H -ligand, excess and choice of unlabelled ligand, incubation time and incubation temperature. The most common choice is to incubate the cytosol with ^3H -estradiol (ER) and ^3H -R 5020 or ^3H -Org 2058 (PgR) at five different concentrations below 10 nM, alone and together with a 100-fold excess diethylstilbestrol (ER) and R 5020 or Org 2058 (PgR) at 0-4°C overnight. The only main difference, at our laboratory compared with these recommendations, is the use of a shorter incubation time (2 hours instead of 16-20 hours). The significance of this difference has been checked in control experiments. In PgR positive breast cancer 20,000xg supernatant samples, the mean difference of PgR recovery was -9.0% (range -32% - +35%) between the two incubation times (2 hours versus 20 hours; n=20). In corresponding experiments for ER, the mean difference in recovery was +11% (range -24 - +80%; n=35). One might argue that the influence on recovery would be different when examining supernatants after 100,000xg centrifugation, which is by far the most commonly used g-value. Therefore, ER and PgR recovery in 100,000xg supernatants after the longer incubation time (16-20 hours) have been compared with that after the shorter incubation time (2 hours), without finding any major differences (n=5).

To summarize the importance of the analytical procedures, it seems reasonable to assume that to obtain as reliable receptor determinations as possible, the laboratory should follow recommendations from international receptor groups, such as the E.O.R.T.C. receptor group (1980). Their recommendations are based on wide experience concerning receptor methods. Differences from these recommendations should be carefully evaluated before changing the assay procedure. Furthermore, it is also of great importance to standardize the measurement and this concerns all steps involved in receptor analysis, some of them, of course, being more important than others. Finally, it is also desirable to participate in control studies, where the results from different laboratories are compared. Our laboratory participates in a quality control study organized by the Swedish Cancer Society.

Choice of method (II). Besides the importance of correct handling of certain analytical steps, the choice of method is also considered to be of significance for evaluation of receptor content. A consensus development meeting on "Steroid Receptors in Breast Cancer" was held at the National Institute of Health in the USA on June 27-29, 1979 (DeSombre et al. 1979). It was recommended at the meeting that the most reliable and reproducible methods for cytosol ER assay appear to be sucrose density gradient sedimentation (SDG) analysis and multiple point dextran-coated charcoal (DCC) assay with Scatchard analysis. Other methods should be validated against these two methods. DCC technique with Scatchard analysis and SDG method are also the most commonly used techniques worldwide. For this reason a comparison has been made between the DCC method with Scatchard analysis and isoelectric focusing (IF), the latter being the most common method used in Sweden for the assay of ER and PgR. This study showed a very good concordance, based on Spearman's rank-correlation test, between the two techniques. The recovery was, however, markedly increased when using DCC with Scatchard analysis instead of IF, which also has been found by Lloyd et al. (1982). IF has also been found to give lesser measurable amounts of ER compared with SDG (Wrange et al. 1976).

As far as the PgR determination with IF in the present study is concerned, the smaller amount of measurable receptor together with, from our experience, more difficulties in the interpretation of the analytical chromatographs compared with the DCC assay and Scatchard analysis, resulted in that the latter technique is still the routine method for PgR at our laboratory. For ER measured with IF, no corresponding difficulties in the interpretation were encountered.

The lower recovery obtained with IF compared with DCC and Scatchard analysis, in paper II, has been suggested to be caused by a dissociation of ³H-ligand from the receptor during the run in the gel, may be in part by the pH gradient. Wrange et al. (1976) have pointed out the possibilities of receptor aggregation and denaturation. Another explanation for the higher recovery obtained with DCC method and Scatchard analysis may be that other saturable, non-receptor binding sites or inadequate receptor forms may be measured with the DCC assay and Scatchard analysis. One important advantage with IF is the smaller amount of tissue material required for receptor assays. This will of course be particularly important when the tumour samples are small and it has also been possible to measure, at least ER, in fine needle aspirates (Lindgren et al. 1980, Silfverswärd et al. 1980). The overall good concordance between IF and DCC method with Scatchard analysis makes it reasonable to conclude that also IF can be considered as a reliable method for ER and PgR.

A new technique, not based on the hormone binding capacity, but rather on the antigenic recognition of ER (ER-EIA = ER enzyme immuno assay) has recently been developed (Greene and Jensen 1982). Investigations at our as well as other laboratories have been started in order to evaluate EIA against the routine techniques of today, in respect of ER recovery, reliability, standardization, handling and expense. Results obtained so far indicate that EIA gives comparable results with present standard techniques, using radio-labelled ligands (Greene and Jensen 1982, Fernö et al. 1985).

Choice of reference parameter (III). The most common reference parameter is protein, but also DNA and wet weight are used. In the present work, a very good correlation was found between these three reference parameters in 235 breast cancer biopsies. Possible explanations for this high concordance might be that 1/ the range of receptor values is much wider compared with the range of protein and DNA and 2/ the protein content (per mg wet weight) showed a good correlation with the corresponding DNA content. The choice of reference parameter may, however, in single cases be important for the classification of receptor status. Namely, the present work has shown that occasional patient will switch from below to above the cut-off point dependent on the reference parameter used. However, in order to evaluate the best parameter in this respect, extensive breast cancer material is certainly needed.

A possible advantage of DNA as reference parameter is indicated, since both ER and PgR contents were correlated with the amount of DNA on wet weight basis but not with the corresponding amount of proteins. An overrepresentation of samples with negative or low receptor values were thus found in a group of tumours with a low DNA content per mg wet weight, indicating the possibility that these samples are so called "false receptor negatives". It would therefore be desirable to evaluate these samples together with the histopathological picture in order to examine if 1/ the cancer cells are sparsely distributed making receptor measurement unreliable or 2/ if the samples represent a certain histopathological type with true low receptor values.

An attempt to evaluate the first point has been made in paper III by taking also the DNA concentration in the homogenate into consideration, which may reflect the concentration of cells and a possible improper dilution of the sample. In accordance with the DNA content expressed per mg wet weight, also the DNA concentration in the homogenate was correlated with the receptor content. An overrepresentation of receptor negative tumours was thus found in the group of tumour samples with a low DNA concentration in the homogenate. But as the DNA concentration in the

homogenate was strongly correlated with DNA/mg wet weight, the present study has not evaluated this point finally.

Great progress in the expression of the receptor content is possible made in the near future through the development of ER immunocytochemical assay (ER-ICA). With ER-ICA it will hopefully be possible to at least semiquantitate the receptor content, to get an impression of the number of cancer cells and finally also to estimate the heterogenous distribution of ER in different cancer cells within the same sample (Greene et al. 1984).

CLINICAL SIGNIFICANCE OF RECEPTOR ASSAYS

Breast cancer (IV).

It is generally accepted that knowledge of receptor content in human breast cancer samples is a prerequisite for determining the optimal treatment of patients with disseminated disease. While 70-75% of those with ER and PgR positive tumours respond to hormonal therapy, only approximately 10% of those lacking both receptors will respond. The latter group will thus probably get more benefit from cytotoxic treatment.

In 1977, Knight and co-workers reported that patients with receptor positive samples had a more favourable prognosis in terms of recurrence free survival (RFS) compared with those with receptor negative samples. This property of receptor as an independent prognostic variable for RFS has been confirmed by a great number of other investigators (Hähnel et al. 1979, Godolphin et al. 1981, Clark et al. 1983). Several other studies have, however, failed to show any correlation between RFS and ER or PgR levels (Hilf et al. 1980 I, Shapiro et al. 1982, Howat et al. 1983, Parl et al. 1984). These contradictory findings may at least partly be explained by a heterogenous patient material in terms of menopausal status (Logan et al. 1982) and lymph node involvement (Kinne et al. 1981, Saez et al. 1984), by a possible administration of adjuvant therapy (e.g. hormonal or cytotoxic) and also by the length of the follow-up time (Hähnel et al. 1979, Aamdal et al. 1984). From the methodological point of view it should be mentioned the importance of the choice of method for receptor assay, the handling of the biopsy material from the operation to the receptor laboratory and last but not least, also the importance of a correct performance of the assay as well as method standardization and quality control. These methodological aspects have been discussed above. The varying findings for receptors as variables of prognostic importance for RFS may also be due to the choice of statistical method (Hilf et al. 1980 I), the number of patients in study (Hilf et al. 1980 I) and the

cut-off level for distinguishing between receptor positive and negative samples (Forrest et al. 1980). The finding that the receptor status also predicts the survival time may be influenced by the choice of therapy for patients with recurrences (Parl et al. 1984). Namely it would be reasonable to assume that patients with receptor positive tumours are usually given hormonal drugs with a greater therapeutic success, than in cases with receptor negative tumours.

The findings in the present study (IV) do not suggest any correlation between RFS and ER, or PgR status in premenopausal patients with the cut-off points commonly applied at our laboratory. However, by choosing higher cut-off points, significant correlation between RFS and ER or PgR was obtained. The importance of the chosen cut-off point has also been shown by Forrest et al. (1980). The relationship between RFS and receptors at the higher cut-off points, was in our study, found to be restricted to premenopausal patients treated with adjuvant cyclophosphamide alone, or in combination with radiotherapy. One explanation for this may be attributed to the endocrine effects of cyclophosphamide, which in the present study have been indicated by amenorrhea for the vast majority of the patients in this treatment groups. As far as the overall survival for premenopausal patients was concerned, significant differences in favour of patients with receptor positive tumours were found. In the present study this finding was explained by a longer survival time after recurrence, which was probably due to the higher frequency and the longer duration of response to endocrine treatment of metastatic disease in patients with receptor positive primary tumours compared with those patients with receptor negative tumours.

In postmenopausal patients not treated with adjuvant tamoxifen, no relationship was found between RFS and receptor status, irrespective of chosen cut-off point. However, those postmenopausal patients treated with adjuvant tamoxifen had a more favourable RFS when their tumour was receptor positive compared with receptor negative. Similar results have been obtained by others (Wallgren et al. 1984, Rose et al. 1985, Stewart and Prescott 1985). Another large trial did, however, not demonstrate any relationship between RFS and ER for patients treated with adjuvant tamoxifen (Baum et al. 1985). The reasons, which are discussed above, may contribute to explain these contradictory findings. Our study have furthermore shown that postmenopausal patients treated with adjuvant tamoxifen and with ER+/PgR+ tumours, had a statistically better RFS ($p=0.04$) compared with those patients with ER+/PgR- tumours. This finding indicates that additional information is obtained by also measuring PgR.

Summing up, the relationship between RFS and receptors has in the present study shown to be influenced by 1/ adjuvant therapy and 2/ choice of cut-off point. The menopausal status may also be of importance. In future it will be interesting to further subgroup the material in respect of lymph nodal involvement and the histopathological picture.

Endometrial carcinoma (V).

The now well established use of steroid receptor status in the clinical management of breast cancer has resulted in the investigation of the value of such measurements in other hormone dependent malignant diseases. One such disease, is endometrial carcinoma, where earlier investigations have pointed out that steroid receptors may have importance for the prognosis of the disease and for the response to progestational therapy (Kauppila et al. 1982, Martin et al. 1983).

About one third of patients with endometrial cancer respond to treatment with gestagens (Kelley and Baker 1961). This response rate is higher for patients with well differentiated (WD) tumours compared with patients with poorly differentiated tumours (Malkasian et al. 1971). The well established relationship between steroid receptor content and response to endocrine treatment in breast cancer patients with disseminated disease makes it, of course, interesting to evaluate if a corresponding relationship also exists in endometrial carcinoma.

The present study of steroid receptor content in endometrial carcinoma was therefore started with the aim to investigate if steroid receptor measurement could provide further valuable clinical information for this disease. The study has demonstrated the presence of ER in all and PgR in 88% of the endometrial cancers. The fraction of PgR positive samples as well as mean ER and PgR concentrations were, similar to other investigators (Kauppila et al. 1982), found to be positively correlated with the degree of tumour differentiation. But in the groups with moderately (MD) and poorly differentiated (PD) tumours, two subgroups could be distinguished - one with low and one with high receptor concentrations. These subgroups did not differ in any other variable studied, including thymidine incorporation rate, plasma estradiol and progesterone concentrations and patient's age. The steroid receptor concentration may therefore be an expression of an important independent biological marker of the tumour and may also be of significance for the selection of patients for endocrine treatment. Patients with MD and PD tumours containing high receptor concentrations may thus, together with those patients with WD tumours, be the most appropriate for hormonal therapy. To further study steroid receptors as independent prognostic indicators, other factors of prog-

nostic importance should be taken into consideration, e.g. myometrial invasion, tumour size, ovarian and distant metastasis.

ANTIESTROGEN BINDING SITES IN BREAST CANCER (VI)

The lack of complete concordance between measurable receptor content in breast cancer samples and the response to hormonal treatment has been the object of intense work. Although several possible explanations for the so-called "false positive" and "false negative" breast cancer receptor assays have been given (for more details, see page 18-20 in Introduction), no generally accepted explanation for this disagreement has as yet been presented.

A further element in this discussion may be the antiestrogen binding site ($\text{AEBS}_{\text{spec}}$), which was discovered by Sutherland et al. (1979). $\text{AEBS}_{\text{spec}}$ binds triphenylethylene antiestrogens (eg. tamoxifen and its metabolites) but not estrogens.

In this work, a basic investigation of this binding site in breast cancer biopsy samples has been carried out. $\text{AEBS}_{\text{spec}}$ could either be measured with tamoxifen or some of its main metabolites (4-OH-tamoxifen or N-d-Me-tamoxifen). But, as these ligands also bind to ER, it is necessary to subtract this binding from the total binding of tamoxifen or its main metabolites, in order to get the concentration of $\text{AEBS}_{\text{spec}}$. Our study has demonstrated that the inhibition by excess non-radioactive estradiol on ^3H -4-OH-tamoxifen binding was better correlated with ER content (measured in the same sample) than if ^3H -tamoxifen was the radioactive tracer. It is therefore suggested that ^3H -4-OH-tamoxifen is a better choice than ^3H -tamoxifen to measure the concentration of $\text{AEBS}_{\text{spec}}$.

The importance of $\text{AEBS}_{\text{spec}}$ for the clinical effects of tamoxifen may depend upon the fact that its concentration was found to be 5-10 times greater than that of ER. Furthermore, the affinity of tamoxifen to $\text{AEBS}_{\text{spec}}$ was stronger than to ER. This difference in affinity as well as in concentration may influence the amount of tamoxifen available for ER. As the anti-tumoural properties of tamoxifen are considered to be mediated by a blockade of ER, these findings may have a bearing on the clinical effects of tamoxifen. It should therefore be of interest to evaluate the clinical effects of tamoxifen together with the concentration of $\text{AEBS}_{\text{spec}}$ as well as that of ER.

As mentioned above, steroid receptor determinations are of well-documented value for the choice of therapy for patients with disseminated breast cancer. They may also be of significance for getting an opinion of the prognosis of the disease and for the choice of adjuvant therapy.

The discovery of ER led also to the idea that estrogens might serve as carrier molecules for certain cytotoxic substances to malignant tumours containing ER, thus decreasing side effects and improving clinical effects (Könyves and Liljekvist 1976). A substance synthesized according to this idea, namely a nornitrogen mustard derivate of estradiol-17 β -phosphate (estramustine phosphate = EMP) has been shown to be effective in the therapy of prostatic carcinoma (Edsmyr et al. 1982). EMP was, however, found only to have positive effects in a smaller number of breast cancer patients (Groupe European Du Cancer Du Sein 1969, Alexander et al. 1979, Dawes 1982) and another study has not been able to demonstrate any binding of EMP to ER (Forsgren et al. 1979). The effect of EMP in prostatic carcinoma has, in later studies, been suggested to be mediated through an estramustine binding protein (EMBP; Björk et al. 1982), first discovered in the ventral prostate of the rat.

In the present study, a similar estramustine binding site (EMBS), possibly identical to EMBP, has been found in 74 (24%) of 306 breast cancer samples. The observed peak in the frequency of EMBS positivity for patients between 50-59 years of age may be explained by the hormonal changes occurring in connection with the menopause.

A significant correlation between EMBS positivity and receptor (ER and PgR) negativity was found for postmenopausal patients, while a tendency to correlation was found for premenopausal patients. This is of particular interest, since patients with receptor negative tumour samples represent a group where the need for new, less toxic treatment is most obvious. It would therefore be of interest to evaluate the clinical effects of EMP in receptor negative postmenopausal breast cancer patients in relation to EMBS status.

Binding sites for estramustine have also been reported in samples from pancreas carcinoma (Andrén-Sandberg et al. 1985) and, at our laboratory, in samples from malignant melanoma (Jönsson et al. 1985). In both of these malignant tumour disorders, as well as in breast cancer, investigations have started in order to evaluate the clinical significance of treatment with EMP.

SUMMARY AND CONCLUSIONS

A method, suitable for routine measurement of estrogen (ER) and progesterone receptors (PgR) in the same sample from breast and endometrial carcinoma, was developed. From the Methodological Part the following can be concluded:

- Some analytical steps (g-value at the homogenate centrifugation and dextran-coated charcoal treatment, used to separate specifically from non-specifically bound and unbound ^3H -ligand) have, been found to be of great importance for receptor recovery. The need of standardization of these steps is obvious.

- Isoelectric focusing (IF) and dextran-coated charcoal (DCC) method with Scatchard analysis demonstrated a very good concordance for the measurement of ER and PgR. The measurable amount of ER and PgR was, however, markedly increased when using DCC with Scatchard analysis instead of IF. In spite of the latter finding it could be concluded that both techniques belong to those methods, which are suitable for routine receptor determinations.

- Three reference parameters (wet weight, protein and DNA) were studied and gave principally the same information about receptor content. A possible advantage of DNA, compared with protein as reference parameter, is indicated by the correlation between DNA and receptor content. An overrepresentation of receptor negativity was then found among samples with a low DNA content (per mg wet weight or ml homogenate). Samples in this group may represent "false receptor negative" samples. The significance of this hypothesis should be evaluated by parallel receptor analyses and histopathological examinations, as well as histochemical receptor assays.

The clinical applications of steroid receptor determinations have, in the present study, been examined for breast cancer (stage II) and endometrial cancer.

- For premenopausal breast cancer patients a relation between a better recurrence free survival (RFS) and high ER and PgR concentrations was found. This relationship seemed to be influenced by adjuvant cyclophosphamide.

- For postmenopausal breast cancer patients, not treated with adjuvant tamoxifen, no relationship between receptor status and RFS was found.

- Those postmenopausal breast cancer patients with receptor positive tumours, treated with adjuvant tamoxifen for one year, with or without postoperative radiotherapy, had a significantly better RFS compared with those patients with receptor negative tumours.

- The correlation between overall survival and receptor status has, in the present study, been explained by the better response for endocrine treatment in breast cancer patients with receptor positive primary tumours, compared with those patients with receptor negative tumours.

- For endometrial carcinoma, the receptor content was correlated with the degree of tumour differentiation. Furthermore, two subgroups were distinguished in poorly (PD) and moderately differentiated (MD) tumour samples, one with high receptor concentrations and one with low receptor concentrations. This may point out a group of MD and PD tumours, where endocrine therapy may be beneficial. These findings also indicate that additional prognostic information may be obtained by the measurement of ER and PgR.

In addition to ER and PgR, other binding sites (antiestrogen binding site (AEBS_{spec}) and estramustine binding site (EMBS)) have been measured in human breast cancer samples.

- AEBS_{spec} was found in almost all samples at 5-10 times the corresponding ER and PgR concentration values. The dissociation constant (K_d) indicates that AEBS_{spec} is a high affinity binding site, similar to steroid receptors. The affinity of tamoxifen to AEBS_{spec} was much stronger than that to ER. The concentration of AEBS_{spec}, in relation to ER content, might be of importance for the clinical effects of tamoxifen.

- EMBS positivity was significantly overrepresented in receptor negative samples from postmenopausal patients. As far as premenopausals were concerned only a tendency to a correlation was obtained. The highest incidence of EMBS positivity was found for patients between 50 and 59 years of age, which was the age group with the lowest incidence of ER and PgR positivity. The effect of estramustine phosphate in the therapy of breast cancer patients should be correlated with the EMBS status.

ACKNOWLEDGEMENTS

Dick Killander, head of the Department of Oncology, for his great interest, generous and encouraging support and for constructive criticism.

Allan Norgren, who introduced me to the field of steroid receptors and scientific work.

Ake Borg for his generous collaboration and help, as well as many good ideas throughout the whole study.

Ulla Johansson and Gunilla Sellberg for excellent technical assistance and a never ending willingness for cooperation in experimental work.

Bo Baldetorp, for interest and support during the study and for his great help in making all practical problems solvable.

Stefan Rydén, for stimulating cooperation.

Members of the South Swedish Breast Cancer Group, without whose help and interest in providing sample material, it would not have been possible to carry out this study.

Claes Tropé, who introduced me to the field of endometrial cancer.

Ingrid Idvall, Karin Tsiobanelis, Per Alm, Larsolof Hafström, Bengt Lindahl and Torgil Möller for collaboration in different parts of the study.

Eva Henriksson for skilful drawing and great help with the lay-out.

Bo Gullberg, Jonas Ranstam and Claes Westrup for statistical advice and computer processing.

Barbara Lindberg for linguistic revision.

My Mother and Father, for their interest and support, and especially my Father also for many valuable discussions in the field of hormones.

Helena, my wife, and Edvard and Oskar, our sons, for creating a pleasant, relaxed atmosphere at home, far away from steroid receptors and binding sites, and further thanks are due to Helena for introducing me in the world of the word processor.

This work was supported by grants from the Swedish Cancer Society, John and Augusta Persson's Foundation for Medical Scientific Research, the Swedish Medical Research Council, Berta Kamprad's Foundation, the Medical Faculty, University of Lund and from Lund's Hospital Research Foundations.

REFERENCES

- Aamdal S., Börmer O., Jörgensen O., Höst H., Eliassen G., Kaalhus O. and Pihl A. Estrogen receptors and long-term prognosis in breast cancer. *Cancer* 53, 1984, 2525-2529.
- Alexander N.CH., Hancock A.K., Masood M.B., Peet B.G., Price J.J., Turner R.L., Stone J. and Ward A.J. Estracyt in advanced carcinoma of the breast: A phase II study. *Clin. Radiol.* 30, 1979, 139-147.
- Alford T.C., Do H.-M., Geelhoed G.W., Tsangaris N.T. and Lippman M.E. Steroid hormone receptors in human colon cancers. *Cancer* 43, 1979, 980-984.
- Allegra J.C., Lippman M.E., Thompson E.B. and Simon R. An association between steroid hormone receptors and response to cytotoxic chemotherapy in patients with metastatic breast cancer. *Cancer Res.* 38, 1978, 4299-4304.
- Allegra J.C., Lippman M.E., Thompson E.B., Simon R., Barlock A., Green L., Huff K.K., Do H.-M.T., Aitken S.C. and Warren R. Relationship between the progesterone, androgen and glucocorticoid receptor and response rate to endocrine therapy in metastatic breast cancer. *Cancer Res.* 39, 1979, 1973-1979.
- Anderson K.M., Phelan J., Marogil M., Hendrickson C. and Economou S. Sodium molybdate increases the amount of progesterone and estrogen receptor detected in certain human breast cancer cytosols. *Steroids* 35 (3), 1980, 273-280.
- Anderson W.A., Kang Y.-H. and DeSombre E.R. Endogenous peroxidase: Specific marker enzyme for tissues displaying growth dependency on estrogen. *J. Cell Biol.* 64, 1975, 668-681.
- Andrén-Sandberg A., Björk P., Gunnarsson P.O. and Ihse I. Uptake and binding of estramustine in nitrosamine-induced exocrine pancreatic cancer in the Syrian golden hamster. Accepted in *Eur. J. Surg. Oncol.* 1985.
- Atkins H., Bulbrook K.D., Falconer M.A., Hayward J.L., MacLean K.S. and Schurr P.H. Ten years' experience of steroid assays in the management of breast cancer. *Lancet* ii, 1968, 1255-1260.
- Barnes D.M., Skinner L.G. and Ribeiro G.G. Triple hormone-receptor assay: A more accurate predictive tool for the treatment of advanced breast cancer? *Br. J. Cancer* 40, 1979, 862-865.
- Baum M., Brinkley D.M., Dossett J.A., McPherson K., Patterson J.S., Rubens R.D., Smiddy F.G., Stoll B.A., Wilson A., Richards D. and Ellis S.H. Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. *Lancet* i, 1985, 836-840.
- Beatson G.T. On the treatment of inoperable cases of carcinoma of the mamma: Suggestions for a new method of treatment, with illustrative cases. *Lancet* ii, 2, 1896, 104-107.
- Björk P., Forsgren B., Gustafsson J.-A., Pousette A. and Högberg B. Partial characterization and "quantitation" of a human prostatic estramustine-binding protein. *Cancer Res.* 42, 1982, 1935-1942.
- Bloom N.D. and Fishman J.H. Tamoxifen treatment failures in hormonally responsive breast cancers. Correlation with steroid receptors. *Cancer* 51, 1983, 1190-1194.

- Blossey H.C., Wander H.E., Koebberling J. and Nagel G.A. Pharmacokinetic and pharmacodynamic basis for the treatment of metastatic breast cancer with high-dose medroxyprogesterone acetate. *Cancer* 54, 1984, 1208-1215.
- Braunsberg H., Carter A.E., James V.H.T. and Jamieson C.W. Studies on the estimation and clinical significance of estrogen receptors in human malignant breast tumors. In: *Estrogen receptors in human breast cancer*. McGuire W.L. et al. (eds). Raven Press, New York 1975, 247-262.
- Burton K.A. Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62, 1956, 315-323.
- Catt K.J. and Dufau M.L. Peptide hormone receptors. *Annu. Rev. Physiol.* 39, 1977, 529-557.
- Chamness G.C. and McGuire W.L. Methods for analyzing steroid receptors in human breast cancer. In: *Breast cancer. Advances in Research and Treatment*. Plenum New York, 3, 1979, 149-197.
- Chang J.C. and Wergowske G. Correlation of estrogen receptors and response to chemotherapy of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) in advanced breast cancer. *Cancer* 48, 1981, 2503-2506.
- Chester Z., Feherty P., Kellie A.E. and Ralphs D.N.L. Estrogen receptor in primary breast tumors in relation to the stage and progression of the disease. In: *Estrogen Receptors in Human Breast Cancer*. McGuire W.L. et al. (eds). Raven Press, New York 1975, 157-174.
- Clark G.M., McGuire W.L., Hubay C.A., Pearson O.H. and Marshall J. S. Progesterone receptors as a prognostic factor in stage II breast cancer. *N. Eng. J. Med.* 309 (22), 1983, 1343-1347.
- Clark J.H., Peck E.J., Schrader W.T. and O'Malley B.W. Estrogen and progesterone receptors: Methods for characterization, quantification, and purification. *Methods in cancer research*. Busch H. (ed). Academic Press, New York 12, 1976, 367-417.
- Clark J.H., Markaverich B., Upchurch S., Eriksson H., Hardin J.W. and Peck Jr E.J. Heterogeneity of estrogen binding sites: Relationship to estrogen receptors and estrogen responses. *Recent. Prog. Horm. Res.* 36, 1980, 89-125.
- Cole M.P., Jones C.T.A. and Todd I.D.H. A new anti-oestrogenic agent in late breast cancer and early clinical appraisal of ICI 46474. *Br. J. Cancer* 25, 1971, 270-275.
- Cooper A.P. *The principles and practice of surgery*. London E. Cox. Lee A. (ed). 1, 1836, 333-335.
- Cowan K. and Lippman M.E. Steroid receptors in breast cancer. *Arch. Intern. Med.* 142, 1982, 363-366.
- Cowan S., Love C. and Leake R.E. The value of determination of nuclear oestrogen receptors in breast cancer biopsies. *Recent. Results. Cancer Res.* 91, 1984, 50-60.
- Daehnfeldt J.L. and Briand P. Determinations of high-affinity gestagen receptors in hormone-responsive and hormone independent GR mouse mammary tumors by and exchange assay. In: *Progesterone Receptors in Normal and Neoplastic Tissues*. McGuire W.L. et al. (eds). Raven Press, New York 1977, 59-69.

Dawes P.J.D.K. A pilot study of Estracyt in advanced breast cancer. Cancer Treat. Rep. 66 (3), 1982, 581-582.

DeSombre E.R., Carbone P.P., Jensen E.V., McGuire W.L., Wells Jr S.A., Wittliff J.L. and Lipsett M.B. Steroid receptors in breast cancer. N. Eng. J. Med. 301 (18), 1979, 1011-1012.

Duffy M.J. and Duffy G. Peroxidase activity as a possible marker for a functional oestradiol receptor in human breast tumours. Biochem. Soc. Trans. 5, 1977, 1738-1739.

Edsmyr F., Andersson L. and Könyves I. Estramustine phosphate (Estracyt): Experimental studies and clinical experience. In: Prostate Cancer, International Perspectives in Urology. Jacobi G.H. and Hohenfeller R. (eds.), Williams & Wilkins, Baltimore 3, 1982, 253-268

Ekman P. The application of steroid receptor assay in human prostate cancer research and clinical management. Anticancer Res. 2, 1982, 163-172.

E.O.R.T.C. Breast Cooperative Group. Revision of the standards for the assessment of hormone receptors in human breast cancer: Report of the Second E.O.R.T.C. Workshop, Held on 16-17 March, 1979, in the Netherlands cancer institute. Eur. J. Cancer 16, 1980, 1513-1515.

Faye J.-C., Jozan S., Redeuilh G., Baulieu E.-E. and Bayard F. Physico-chemical and genetic evidence for specific antiestrogen binding sites. Proc. Natl. Acad. Sci. USA 80, 1983, 3158-3162.

Feherty P., Farrer-Brown G. and Kellie A.E. Oestradiol receptors in carcinoma and benign disease of the breast: An in vitro assay. Br. J. Cancer 25, 1971, 697-710.

Feil P.D., Glasser S.R., Toft D.O. and O'Malley B.W. Progesterone binding in the mouse and rat uterus. Endocrinology 91, 1972, 738-746.

Fernö M., Borg Å. and Sellberg G. Enzyme immuno assay of estrogen receptor (ER) in breast cancer biopsy samples; a comparison with isoelectric focusing and detection of ER during tamoxifen treatment. Submitted for publication.

Fisher B., Redmond C., Brown A., Wickerham D.L., Wolmark N., Allegra J., Escher G., Lippman M.L., Savlov E., Wittliff J. and Fischer E.R. with the contributions of Plotkin D., Bowman D., Wolter J., Bornstein R., Desser R., Frelick R. and other NSABP Investigators. Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. J. Clin. Oncol. 1 (4), 1983, 227-241.

Folca P.J., Glascock R.F. and Irvine W.T. Studies with tritium-labelled hexoestrol in advanced breast cancer. Lancet ii, 1961, 796-798.

Forrest A.P.M., Black R.B., Humeniuk V. et al. Preoperative assessment and staging of breast cancer: preliminary communication. J. Royal Soc. Med. 73, 1980, 561-566.

Forsgren B., Björk P., Carlström K., Gustafsson J.-Å., Pousette A. and Högborg B. Purification and distribution of a major protein in rat prostate that binds estramustine, a nitrogen mustard derivative of estradiol-17 β . Proc. Natl. Acad. Sci. USA 76, 1979, 3149-3153.

Friedman M., Lagios M., Markowitz A., Jones H., Resser K. and Hoffman P. Estradiol (ER) and progesterone receptors (PR) in ovarian cancer - clinical and pathological correlation. Clin. Res. 27 (1), 1979, 385 A.

Gairard B., Calderoli H., Keiling R., Renaud R., Bellocq J.P. and Koehl

C. Cancer cell counts and validity of steroid receptor determinations in breast cancer. *Lancet* ii, 1981, 1419.

Gale K.E. Treatment of advanced breast cancer with aminoglutethimide: A 14-year experience. *Cancer Res.* (suppl.) 42, 1982, 3389s-3396s.

Garola R.E. and McGuire W.L. An improved assay for nuclear estrogen receptor in experimental and human breast cancer. *Cancer Res.* 37, 1977, 3333-3337.

Garola R.E. and McGuire W.L. A hydroxylapatite micromethod for measuring estrogen receptor in human breast cancer. *Cancer Res.* 38, 1978, 2216-2220.

Glascok R.F. and Hoekstra W.G. Selective accumulation of tritium-labelled hexoestrol by the reproductive organs of immature female goats and sheep. *Biochem. J.* 72, 1959, 673-682.

Godolphin W., Elwood J.M. and Spinelli J.J. Estrogen receptor quantitation and staging as complementary prognostic indicators in breast cancer: A study of 583 patients. *Int. J. Cancer* 28, 1981, 677-683.

Goldberg N.D. and Haddox M.K. Cyclic GMP metabolism and involvement in biological regulation. *Annu. Rev. Biochem.* 46, 1977, 823-896.

Goldenberg I.S., Waters M.N., Ravdin R.S., Ansfield F.J. and Segaloff A. Androgenic therapy for advanced breast cancer in women: A report of the Cooperative Breast Cancer Group. *J. Am. Med. Ass.* 223 (11), 1973, 1267-1268.

Gorden P., Carpentier J.-L., Freychet P. and Orci L. Internalization of polypeptide hormones. Mechanism, intracellular localization and significance. *Diabetologia* 18, 1980, 263-274.

Gorski J., Toft D., Shyamala G., Smith D. and Notides A. Hormone receptors: Studies on the interaction of estrogen with the uterus. *Prog. Horm. Res.* 24, 1968, 45-80.

Gorski J. and Gannon F. Current models of steroid hormone action: A critique. *Annu. Rev. Physiol.* 38, 1976, 425-450.

Greene G.L. and Jensen E.V. Monoclonal antibodies as probes for estrogen receptor detection and characterization. *J. Steroid Biochem.* 16, 1982, 353-359.

Greene G.L., Sobel N.B., King W.J. and Jensen E.V. Immunochemical studies of estrogen receptors. *J. Steroid Biochem.* 20 (1), 1984, 51-56.

Greenway B., Iqbal M.J., Johnson P.J. and Williams R. Oestrogen receptor proteins in malignant and fetal pancreas. *Br. Med. J.* 283, 1981, 751-753.

Gross G.E., Clark G.M., Chamness G.C. and McGuire W.L. Multiple progesterone receptor assays in human breast cancer. *Cancer Res.* 44, 1984, 836-840.

Groupe Europeen Du Cancer Du Sein. Essai clinique du phénol bis (2-chloro-éthyl) carbamate d'oestradiol dans le cancer mammaire en phase avancée. *Eur. J. Cancer* 5, 1969, 1-4.

Gustafsson J.-Å., Gustafsson S.A., Nordenskjöld B., Okret S., Silfver-
sward C. and Wrangé Ö. Estradiol receptor analysis in human breast cancer tissue by isoelectric focusing in polyacrylamide gel. *Cancer Res.* 38, 1978, 4225-4228.

Haddow A., Watkinson J.M. and Paterson E. Influence of synthetic oestro-

gens upon advanced malignant disease. Br. Med. J. 1944, 393-398.

Harland R.N.L., Barnes D.M., Howell A., Ribeiro G.G., Taylor J. and Sellwood R.A. Variation of receptor status in cancer of the breast. Br. J. Cancer 47, 1983, 511-515.

Harris A.L., Powles T.J. and Smith I.E. Aminoglutethimide in the treatment of advanced postmenopausal breast cancer. Cancer Res. (suppl.) 42, 1982, 3405s-3408s.

Hawkins R.A., Hill A. and Freedman B. A simple method for the determination of oestrogen receptor concentrations in breast tumours and other tissues. Clinica Chimica Acta 64, 1975, 203-210.

Haynes Jr.R.C., Sutherland E.W. and Rall T.W. The role of cyclic adenylic acid in hormone action. Recent. Prog. Horm. Res. 16, 1960, 121-138.

Henderson I.C. and Canellos G.P. Cancer of the breast. The past decade (Second of two parts). N. Engl. J. Med. 302, 1980, 78-90.

Hilf R., Feldstein M.L., Gibson S.L. and Savlov E.D. The relative importance of estrogen receptor analysis as a prognostic factor for recurrence or response to chemotherapy in women with breast cancer. Cancer 45, 1980, I, 1993-2000.

Hilf R., Feldstein M.L., Savlov E.D., Gibson S.L. and Seneca B. The lack of relationship between estrogen receptor status and response to chemotherapy. Cancer 46, 1980 II, 2797-2800.

Holt J.A., Caputo T.A., Kelly K.M., Greenwald P. and Chorost S. Estrogen and progestin binding in cytosols of ovarian adenocarcinomas. Obstet & Gynecol. 53 (1), 1979, 50-58.

Horwitz K.B. and McGuire W.L. Specific progesterone receptors in human breast cancer. Steroids 25 (4), 1975, 497-505.

Horwitz K.B. Is a functional estrogen receptor always required for progesterone receptor induction in breast cancer? J. Steroid Biochem. 15, 1981, 209-217.

Howat J.M.T., Barnes D.M., Harris M. and Swindell R. The association of cytosol oestrogen and progesterone receptors with histological features of breast cancer and early recurrence of disease. Br. J. Cancer 47, 1983, 629-640.

Huggins C. and Bergenstal D.M. Inhibition of human mammary and prostatic cancers by adrenalectomy. Cancer Res. 12, 1952, 134-141.

Hähnel R. and Twaddle E. Factors that may influence the estradiol receptor assay in human tissues: Sex hormone binding globulin and endogenous steroids. J. Steroid Biochem. 10, 1979, 95-98.

Hähnel R., Woodings T. and Vivian A.B. Prognostic value of estrogen receptors in primary breast cancer. Cancer 44, 1979, 671-675.

Iqbal M.J., Wilkinson M.L., Johnson P.J. and Williams R. Sex steroid receptor proteins in foetal, adult and malignant human liver tissue. Br. J. Cancer 48, 1983, 791-796.

Janssens J.P.H., Bonte J., Drochmans A., Mulier J., Rutten J., Wittevro-n-gel C. and deLoecker W. Effect of presurgical radiotherapy on the steroid receptor concentrations in primary breast carcinoma. Eur. J. Cancer 17 (6), 1981, 659-664.

Jensen E.V. Studies of growth phenomena using tritium-labelled steroids.

Proceedings of the fourth international congress of biochemistry in Vienna 1958. London Pergamon Press 1960, 119.

Jensen E.V. and Jacobson H.I. Fate of steroid estrogens in target tissues. In: Biological Activities of Steroids in Relation to Cancer. Pincus G. and Vollmer E. (eds). Academic Press New York and London, 1960, 161-178.

Jensen E.V. and Jacobson H.I. Basic guides to the mechanism of estrogen action. Recent. Prog. Horm. Res. 17. 1962, 387-414.

Jensen E.V., DeSombre E.R. and Jungblut P.W. Estrogen receptors in hormone-responsive tissues and tumors. In: Endogenous Factors Influencing Host-Tumor Balance. Wissler R.W., Dao T.L. and Wood S. (eds). University of Chicago Press 1967, 15-30.

Jensen E.V., Suzuki T., Kawashima T., Stumpf W.E., Jungblut P.W. and DeSombre E.R. A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Natl. Acad. Sci. USA 59, 1968, 632-639.

Jensen E.V., Block G.E., Smith S., Kyser K. and DeSombre E.R. Estrogen receptors and breast cancer response to adrenalectomy. Natl. Cancer Inst. Monogr. 34, 1971, 5-79.

Jensen E.V. and DeSombre E.R. Mechanism of action of the female sex hormones. Ann. Rev. Biochem. 41, 1972, 203-230.

Jensen E.V. and DeSombre E.R. Estrogen receptor interaction. Estrogenic hormones effect transformation of specific receptor proteins to a biochemically functional form. Science 182, 1973, 126-134.

Jensen E.V., Polley T.Z., Smith S., Block G.E., Ferguson D.J. and DeSombre E.R. Prediction of hormone dependency in human breast cancer. In: Estrogen Receptors in Human Breast Cancer. McGuire W.L. et al. (eds). Raven Press New York, 1975, 37-55.

Jensen E.V., Smith S. and DeSombre E.R. Hormone dependency in breast cancer. J. Steroid Biochem. 7, 1976, 911-917.

Jonat W., Maass H., Stolzenbach G. and Trams G. Estrogen receptor status and response to polychemotherapy in advanced breast cancer. Cancer 46. 1980, 2809-2813.

Jordan V.C. Comparative antioestrogen action in experimental breast cancer. In: Hormones and Cancer. Adv. Exp. Med. Biol. 138, 1982, 165-178.

Jönsson P.E., Ingvar C., Borg Å. and Fernö M. A binding site for estramustin in malignant melanoma. Abstract, 14th international congress of chemotherapy. Osaka, Japan, 1985.

Karr J.P., Pontes J.E., Schneider S., Sandberg A.A. and Murphy G.P. Clinical aspects of steroids hormone receptors in human renal cell carcinoma. J. Surg. Oncol. 23, 1983, 117-124.

Katzenellenbogen B.S., Bhakoo H.S., Ferguson E.R., Lan N.C., Tatee T., Tsai T.-L.S. and Katzenellenbogen J.A. Estrogen and antiestrogen action in reproductive tissues and tumors. Recent Prog. Horm. Res. 35, 1979, 259-300.

Kaupilla A., Kujansuu E. and Vihko R. Cytosol estrogen and progestin receptors in endometrial carcinoma of patients treated with surgery, radiotherapy, and progestin. Clinical correlates. Cancer 50, 1982, 2157-2162.

- Kelley R.M. and Baker W.H. Progestational agents in the treatment of carcinoma of the endometrium. *N. Eng. J. Med.* 264 (5), 1961, 216-222.
- Kiang D.T., Frenning D.H., Goldman A.I., Ascensao V.F. and Kennedy B.J. Estrogen receptors and responses to chemotherapy and hormonal therapy in advanced breast cancer. *N. Engl. J. Med.* 299 (24), 1978, 1330-1334.
- King R.J.B. Clinical relevance of steroid-receptor measurements in tumours. *Cancer Treat. Rev.* 2, 1975, 273-293.
- King R.J.B., Barnes D.M., Hawkins R.A., Leake R.E., Maynard P.V. and Roberts M.M. Measurement of oestradiol receptors by five institutions on common tissue samples. *Br. J. Cancer* 38, 1978, 428-430.
- King W.J. and Greene G.L. Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* 307, 1984, 745-747.
- Kinne D.W., Ashikari R., Butler A., Menendez-Botet C., Rosen P.P. and Schwartz M. Estrogen receptor protein in breast cancer as a predictor of recurrence. *Cancer* 47, 1981, 2364-2367.
- Knight W.A., Livingston R.B., Gregory E.J. and McGuire W.L. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res.* 37, 1977, 4669-4671.
- Koenders A. and Thorpe S.M. Standardization of steroid receptor assays in human breast cancer - I. Reproducibility of estradiol and progesterone receptor assays. *Eur. J. Cancer Clin. Oncol.* 19, 1983 I, 1221-1229.
- Koenders A. and Thorpe S.M. Standardization of steroid receptor assays in human breast cancer - II. Samples with low receptor content. *Eur. J. Cancer Clin. Oncol.* 19, 1983 II, 1467-1472.
- Korenman S.G. Radio-ligand binding assay of specific estrogens using a soluble uterine macromolecule. *J. Clin. Endocrinol. Metab.* 28, 1968, 127-130.
- Korenman S.G. and Dukes B.A. Specific estrogen binding by the cytoplasm of human breast carcinoma. *J. Clin. Endocrinol. Metab.* 30, 1970, 639-645.
- Könyves I. and Liljekvist J. The steroid molecule as a carrier of cytotoxic groups. *International Congress Series No. 375. Biological Characterization of Human Tumours.* Davis W. and Maltoni C. (eds). 3, 1976, 98-105.
- Laing L., Calman K.C., Smith M.G., Smith D.C. and Leake R.E. Nuclear oestrogen receptors and treatment of breast cancer. *Lancet* ii, 1977, 168-169.
- Leake R.E., Laing L., Calman K.C., MacBeth F.R., Crawford D. and Smith D. C. Oestrogen receptor status and endocrine therapy of breast cancer: Response rates and status stability. *Br. J. Cancer* 43, 1981, 59-66.
- Lindgren A., Sällström J. and Adami H.-O. Fine needle biopsy in estrogen receptor determination in breast cancer. In: *Breast Cancer, experimental and clinical aspects.* Mouridsen H.T. and Palshof T. (eds). Pergamon Press Oxford and New York 1980, 67-69.
- Lippman M.E., Bolan G., Monaco M., Pinkus L. and Engel L. Model systems for the study of estrogen action in tissue culture. *J. Steroid Biochem.* 7, 1976, 1045-1051.
- Lippman M.E., Huff K., Bolan G. and Neifeld J.P. Interactions of R 5020 with progesterone and glucocorticoid receptors in human breast cancer and peripheral blood lymphocytes in vitro. In: *Progesterone Receptors in*

Normal and Neoplastic Tissues. McGuire W.L. et al. (eds). Raven Press New York, 1977, 193-210.

Lippman M.E., Allegra J.C., Thompson E.B., Simon R., Barlock A., Green L., Huff K.K., Do H.-M.T., Aitken S.C. and Warren R. The relation between estrogen receptors and response rate to cytotoxic chemotherapy in metastatic breast cancer. N. Engl. J. Med. 298 (22), 1978, 1223-1228.

Lloyd E.J., Barnes D.M. and Skinner L.G. Isoelectric focusing of oestradiol receptor protein from human mammary carcinoma - a comparison with dextran coated charcoal assay. J. Steroid Biochem. 16, 1982, 239-244.

Logan L.A., Cripps M.C., Hirte W.E. and Rapp E.F. The estrogen receptor test: a prognostic tool in primary breast cancer. Can. J. Surg. 25 (5), 1982, 581-584.

Lowry O.H., Rosebrough N.J., Farr L. and Randall R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 1951, 265-275.

Ludwig Breast Cancer Study Group. Randomised trial of chemo-endocrine therapy, endocrine therapy, and mastectomy alone in postmenopausal patients with operable breast cancer and axillary node metastasis. Lancet i, 1984, 1256-1260.

Luft R. and Olivecrona H. Experiences with hypophysectomy in man. J. Neurosurg. 10, 1953, 301-316.

Löber J., Rose C., Salimtschik M. and Mouridsen H.T. Treatment of advanced breast cancer with progestins. Acta Obstet. Gynecol. Scand. Suppl. 101, 1981, 39-46.

Maass H., Jonat W., Stolzenbach G. and Trams G. The problem of nonresponding estrogen receptor-positive patients with advanced breast cancer. Cancer 46, 1980, 2835-2837.

MacCorquodale D.W., Thayer S.A. and Doisy E.A. The isolation of the principal estrogenic substance of liquor folliculi. J. Biol. Chem. 115, 1936, 435-448.

Malkasian G.D., Decker D.G., Mussey E. and Johnson C.E. Progestogen treatment of recurrent endometrial carcinoma. Amer. J. Obst. Gynec. 110 (1), 1971, 15-23.

Martin J.D., Hähnel R., McCartney A.J. and Woodings T.L. The effect of estrogen receptor status on survival in patients with endometrial cancer. Am. J. Obstet. Gynecol. 147 (3), 1983, 322-324.

Martin P.M., Rolland P.H., Jacquemier J., Rolland A.M. and Toga M. Routine analysis of multiple steroid receptors in human breast cancer - I - technological features. Biomedicine 28, 1978, 278-287.

Martuza R.L., MacLaughlin D.T. and Ojemann R.G. Specific estradiol binding in schwannomas, meningiomas, and neurofibromas. Neurosurgery 9 (6), 1981, 665-671.

McCarty K.S.Jr., Wortman J., Stowers S., Lubahn D.B., McCarty K.S. Sr. and Seigler H.F. Sex steroid receptor analysis in human melanoma. Cancer 46 (6), 1980, 1463-1470.

McGuire W.L. and DeLaGarza M. Improved sensitivity in the measurement of estrogen receptor in human breast cancer. J. Clin. Endocrin. Metab. 37, 1973, 986-989.

McGuire W.L. Steroid receptors in human breast cancer. Cancer Res. 38, 1978, 4289-4291.

- McGuire W.L. An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. In: Hormones and Cancer. Iacobelli S. et al. (eds). Raven Press New York, 1980, 337-343.
- Mortimer J., Reimer R., Greenstreet R., Groppe C. and Bukowski R. Influence of estrogen receptor status on response to combination chemotherapy for recurrent breast cancer. Cancer Treat. Rep. 65 (9-10), 1981, 763-766.
- Mouridsen H. and Palshof T. Tamoxifen in advanced breast cancer. Cancer Treat. Rev. 5, 1978, 131-141.
- Nathanson I.T. Clinical investigative experience with steroid hormones in breast cancer. Cancer 5, 1952, 754-762.
- Neifeld J.P. and Lippman M.E. Steroid hormone receptors and melanoma. J. Invest. Dermatol. 74 (6), 1980, 379-381.
- van Netten J.P., Algard F.T., Montessori G. and Weare B. Electrophoretic assay of specific estrogen receptors: A contribution to methodology. Clin. Chem. 23 (11), 1977, 2059-2065.
- van Netten J.P., Algard F.T., Coy P., Carlyle S.J., Brigden M.L., Thornton K.R. and To M.P. Estrogen receptor assay on breast cancer microsamples. Implications of percent carcinoma estimation. Cancer 49, 1982, 2383-2388.
- Nordenskjöld B. Steroid hormone receptors and endocrine management of mammary carcinoma. Acta Med. Scand. 204, 1978, 1-3.
- Norgren A., Sällström J.F. and Lindgren A. Variation of pH of mammary tumour cytosols: Potential influence on steroid receptor assay. Anticancer Res. In press. 1985.
- Panko W.B., Watson C.S. and Clark J.H. The presence of a second, specific estrogen binding site in human breast cancer. J. Steroid Biochem. 14, 1981, 1311-1316.
- Parikh I., Anderson W.L. and Neame P. Identification of high affinity estrogen binding sites in calf uterine microsomal membranes. J. Biol. Chem. 255 (21), 1980, 10266-10270.
- Parl F.F., Schmidt B.P., Dupont W.D. and Wagner R.K. Prognostic significance of estrogen receptor status in breast cancer in relation to tumor stage, axillary node metastasis, and histopathologic grading. Cancer 54, 1984, 2237-2242.
- Pearson O.H., Ray B.S., Harrold C.C., West C.D., Li M.C., Maclean J.P. and Lipsett M.B. Hypophysectomy in treatment of advanced cancer. J. Am. Med. Ass. 161 (1), 1956, 17-21.
- Pietras R.J. and Szego C.M. Estrogen receptors in uterine plasma membrane. J. Steroid Biochem. 11, 1979, 1471-1483.
- Powell B.L., DeLaGarza M., Clark G.M. and McGuire W.L. Estrogen receptor measurement in low-protein breast cancer cytosols. A modified charcoal technique. Breast Cancer Res. Treat. 1, 1981, 33-35.
- Raam S., Gelman R., Cohen J.L. with the assistance of Bacharach A., Fishchinger A.J., Jacobson H.I., Keshgegian A.A., Konopka S.J. and Wittliff J.L. Estrogen receptor assay: Interlaboratory and intralaboratory variations in the measurement of receptors using dextran coated charcoal technique: A study sponsored by E.C.O.G. Eur. J. Cancer 17, 1981, 643-649.
- Ralet P.G.F.A.H. and Brombacher P.J. The use of coated charcoal in the

determination of oestrogen receptor activity. *Europ. J. Nucl. Med.* 6, 1981, 159-162.

Raynaud J.-P. R 5020, a tag for the progestin receptor. In: *Progesterone Receptors in Normal and Neoplastic Tissues*. McGuire W. L. et al. (eds). Raven Press New York, 1977, 9-21.

Raynaud J.-P., Martin P.M., Bouton M.-M. and Ojasoo T. 11β -Methoxy-17-ethynyl-1,3,5,(10)-estratriene-3,17 β -diol (moxestrol), a tag for estrogen receptor binding sites in human tissues. *Cancer Res.* 38, 1978, 3044-3050.

Rice R.G., Madray S., Rocchio R. and Vaughn C.B. The effect of dithiothreitol on the estrogen receptor. *Anticancer Res.* 2, 1982, 141-144.

Rillema J.A. Mechanism of prolactin action. *Fed. Proc.* 39 (8), 1980, 2593-2598.

Rose C., Thorpe S.M., Andersen K.W., Pedersen B.V., Mouridsen H.T., Blichert-Toft M. and Rasmussen B.B. Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. *Lancet* i, 1985, 16-19.

Rosen S.T., Maciorowski Z., Wittlin F., Epstein A.L., Gordon L.I., Kies M.S., Kucuk O., Kwaan H.C., Vriesendorp H., Winter J.N., Fors E. and Molteni A. Estrogen receptor analysis in chronic lymphocytic leukemia. *Blood* 62 (5), 1983, 996-999.

Saez S., Martin P.M. and Chouvet C.D. Estradiol and progesterone receptor levels in human breast adenocarcinoma in relation to plasma estrogen and progesterone levels. *Cancer Res.* 38, 1978, 3468-3473.

Saez S. and Chouvet C. Evidence for high affinity ^3H -tamoxifen cytosol binding protein (s) in a human breast cancer cell line MDA-MB 231 devoid of estrogen receptors. *Proc. Am. Assoc. Cancer Res.* 23, 1983, 710.

Saez S., Cheix F. and Mayer M. Estrogen and Progesterone receptors as prognostic factors in early breast cancer. *Recent Results Cancer Res.* 91, 1984, 192-198.

Scatchard G. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51, 1949, 660-672.

Seematter R.J., Hoffman P.G., Kuhn R.W., Lockwood L.C. and Siiteri P. K. Comparison of (^3H) progesterone and ($6,7\text{-}^3\text{H}$)-17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione for the measurement of progesterone receptors in human malignant tissue. *Cancer Res.* 38, 1978, 2800-2806.

Seibert K. and Lippman M.E. Hormone receptors in breast cancer. *Clin. Oncol.* 1 (3), 1982, 735-794.

Shapiro C.M., Schifeling D., Bitran J.D., Desser R.K., Rochman H., Michel A., Shapiro R., Evans R., Kozloff M.F., Recant W. and Billings A.A. Prognostic value of the estrogen receptor level in pathologic stage I and II adenocarcinoma of the breast. *J. Surg. Oncol.* 19, 1982, 119-121.

Siiteri P.K. Steroid hormones and endometrial cancer. *Cancer Res.* 38, 1978, 4360-4366.

Silfverswärd C., Gustafsson J.-A., Gustafsson S.A., Nordenskjöld B., Wallgren A. and Wrangé Ö. Estrogen receptor analysis on fine needle aspirates and on histologic biopsies from human breast cancer. *Eur. J. Cancer* 16, 1980, 1351-1357.

Stedman K.E., Moore G.E. and Morgan R.T. Estrogen receptor proteins in diverse human tumors. *Arch. Surg.* 115, 1980, 244-248.

- Sterling K. Thyroid hormone action at the cell level. (First of two parts). N. Engl. J. Med. 300 (3), 1979, 117-123.
- Stewart H.J. and Prescott R. Adjuvant tamoxifen therapy and receptor levels. Lancet i, 1985, 573.
- Stoll B.A. "False-positive" oestrogen-receptor assay in breast cancer. Lancet ii, 1977, 296-297.
- Sutherland R.L. and Foo M.S. Differential binding of antiestrogens by rat uterine and chick oviduct cytosol. Biochem. Biophys. Res. Comm. 91 (1), 1979, 183-191.
- Sutherland R.L. and Murphy L.C. The binding of tamoxifen to human mammary carcinoma cytosol. Eur. J. Cancer. 16, 1980 I, 1141-1148.
- Sutherland R.L., Murphy L.C., San Foo M., Green M.D. and Whybourne A.M. High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. Nature 288, 1980 II, 273-275.
- Terenius L. Selective retention of estrogen isomers in estrogen - dependent breast tumors of rats demonstrated by in vitro methods. Cancer Res. 28, 1968, 328-337.
- Teulings F.A.G., van Gilse H.A., Henkelman M.S., Portengen H. and Alexieva-Figusch J. Estrogen, androgen, glucocorticoid, and progesterone receptors in progestin - induced regression of human breast cancer. Cancer Res. 40, 1980, 2557-2561.
- Toft D. and Gorski J. A receptor molecule for estrogens: Isolation from the rat uterus and preliminary characterization. Proc. Nat. Acad. Sci. USA 55, 1966, 1574-1581.
- Vass M.A. and Green B. Effect of progesterone pretreatment on the uptake of estradiol-17 β by the uterine epithelium of the rat. Cell Tissue Res. 202, 1979, 171-175.
- Volk H., Deupree R.H., Goldenberg I.S., Wilde R.C., Carabasi R.A. and Escher G.C. A dose response evaluation of delta-1-testololactone in advanced breast cancer. Cancer 33, 1974, 9-13.
- Wallgren A., Baral A., Carstensen J., Friberg S., Glas U., Hjalmar M.L., Kaigas M., Nordenskjöld B., Skoog L., Theve N.O. and Wilking N. Adjuvant Nolvadex treatment in postmenopausal breast cancer, the Stockholm experience. Abstract, 2nd International symposium on Anti-Hormones in Breast Cancer. West Berlin, W-Germany, 1984.
- Welshons W.V., Lieberman M.E. and Gorski J. Nuclear localization of unoccupied oestrogen receptors. Nature 307, 1984, 747-749.
- Westerberg H., Nordenskjöld B., Wrange Ö., Gustafsson J.-Å., Humla S., Theve N.O., Silfverswärd C. and Granberg P.-O. Effect of antiestrogen therapy on human mammary carcinomas with different estrogen receptor contents. Eur. J. Cancer 14, 1978, 619-622.
- Williams R.H. Textbook of Endocrinology. Washington, Saunders W.B. Company 1981.
- Wittliff J.L. Steroid - binding proteins in normal and neoplastic mammary cells. In: Methods Cancer Res. 11, 1975, 293-354.
- Wittliff J.L., Fisher B. and Durant J.R. Establishment of uniformity in steroid receptor analyses used in cooperative clinical trials of breast cancer treatment. Recent Results Cancer Res. 71, 1980, 197-206.

Wittliff J.L., Wheeler Feldhoff P., Fuchs A. and Wiehle R.D. Polymorphism of estrogen receptors in human breast cancer. In: Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action. Soto R.J. (ed). New York Liss cop. 1981.

Wittliff J.L. Steroid - hormone receptors in breast cancer. Cancer 53, 1984, 630-643.

Wolf R.M., Schneider S.L., Pontes J.E., Englander L., Karr J.P., Murphy G. P. and Sandberg A.A. Estrogen and progestin receptors in human prostatic carcinoma. Cancer 55, 1985, 2477-2481.

Wrangé Ö., Nordenskjöld B., Silfverswärd C., Granberg P.O. and Gustafsson J.-Å. Isoelectric focusing of estradiol receptor protein from human mammary carcinoma - a comparison to sucrose gradient analysis. Europ. J. Cancer 12, 1976, 695-700.

Wrangé Ö., Nordenskjöld B. and Gustafsson J.-Å. Cytosol estradiol receptor in human mammary carcinoma: An assay based on isoelectric focusing in polyacrylamide gel. Anal. Biochem. 85, 1978, 461-475.

Wrangé Ö., Humla S., Ramberg I., Gustafsson S.A., Skoog L., Nordenskjöld B. and Gustafsson J.-Å. Progestin-receptor analysis in human breast cancer cytosol by isoelectric focusing in slabs of polyacrylamide gel. J. Steroid Biochem. 14, 1981. 141-148.

Young P.C.M. and Cleary R.E. Characterization and properties of progesterone - binding components in human endometrium. J. Clin. Endocrinol. Metab. 39 (3), 1974, 425-439.

Zänker K.S., Prokscha G.W. and Blümel G. Plasma membrane - integrated estrogen receptors in breast tissue: Possible modulator molecules for intracellular hormone level. J. Cancer. Res. Clin. Oncol. 100, 1981, 135-148.

Improved Method for Assay of Estradiol and Progesterone Receptors with Special Reference to Breast Cancer

ALLAN NORGRÉN¹, ÅKE BORG¹, MÅRTEN FERNÖ¹, ULLA JOHANSSON¹, BENGT LINDAHL²
and KARIN TSIOBANELIS¹

¹Department of Oncology and ²Department of Obstetrics and Gynecology, University Hospital, S-221 85 Lund, Sweden

Abstract. *Isoelectric focusing of estradiol receptor (ER) and multiple point dextran-coated charcoal (DCC) technique for progesterone receptor (PgR) were adapted for assay of both receptors in the same cytosol sample. Technical improvements and increased recovery of receptors were achieved. 1.0 fmol/ml cytosol of ER may be detected; sensitivity of PgR was 5-10 fmol/ml cytosol. Quantitation of ER was made in 414 (95%) out of 434 consecutive breast cancers. Inclusion of isoelectric focusing records showing only qualitative indication for presence of ER resulted in 98% ER containing tumours, supporting the aspect that strictly ER negative tumours are a rarity. Implications for therapy in disseminated disease are discussed.*

Several methods are available for the measurement of estrogen (ER) or progesterone (PgR) receptors in biological material (1). As a number of different steps are involved in the analytical procedure, loss or reduced recovery of the receptor may occur. In breast and uterine cancer, loss of ER means decreased predictive value of the receptor measurements, since it has been claimed that not only the presence but also the amount of the receptor is related to tumour response to hormonal therapy (2). Optimization of the assay conditions are therefore of utmost importance.

The predictive value of receptor assays in breast disease seems to be improved when ER as well as PgR concentrations are available (3). Methods developed for the estimation of both receptors in the same biopsy sample are of potential advantage, since (a) less amount of tissue may be needed; (b) the receptor profile will be more representative for the biopsy material under analysis; (c) the handling of the samples will be more uniform; (d) the assays will be less time consuming, and (e) both ER and PgR may be measured in an increased proportion of delivered samples.

For routine purposes assays of receptor activity should be specific, simple and should not include the use of expensive laboratory equipment (4). In our laboratory ER and PgR in breast tumours have hitherto been analyzed with the isoelectric focusing (IF) (5) and the multiple point dextran-

coated charcoal (DCC) techniques, respectively, in separate tumour samples.

The question was raised whether (a) ER and PgR could be measured in the same cytosol sample with the potential advantages mentioned above and (b) ER and PgR recovery could be increased by simple modifications in certain steps of the assay. In this report, a procedure resulting in high recovery of ER is described for the routine analysis of breast cancer specimen.

Materials and Methods

Tissue. Normal tissue was removed from mature rabbit or rat uteri. Tumour tissue was obtained from human breast or uterine cancers at operation. The material was freed from fat, blood clot etc., minced, frozen and kept at either -70°C or in liquid nitrogen until analysis.

Analytical procedures

Chemicals. All chemicals were of analytical grade. Disodium-EDTA, KCl, CaCl₂, sodium azide and dithiothreitol were obtained from Merck, Darmstadt; Tris [2-amino-2-(hydroxymethyl)-propane-1,3-diol] and glycerol from BDH-Chemicals Ltd, Parkstone, England; 2-mercaptoethanol from SERVA Feinbiochemica, Heidelberg, West Germany; activated charcoal (Norit A) and trypsin from Sigma Chemicals Co., St Louis, Mo; Dextran T 70 from Pharmacia Fine Chemicals, Uppsala, Sweden.

Ligands. ³H-estradiol (85-115 Ci/mmol) and ³H-promegestone (³H-R 5020; 81-87 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. and purified on a Sephadex G-200 column (Pharmacia, Fine Chemicals, Uppsala, Sweden). After purification the steroids were stored in 99.5% ethanol at -20°C at a concentration of 1-2 µCi/10 µl. Non-radioactive estradiol was a gift from LEO Pharmaceutical Company, Helsingborg, Sweden; non-radioactive R 5020 was obtained from New England Nuclear.

Buffer solutions. TEM (Tris-HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM; pH 7.4); TEK (Tris-HCl 10 mM, EDTA 1.0 mM, KCl 50 mM; pH 7.4); TE (Tris-HCl 10 mM, EDTA 1.5 mM; pH 7.4). Sodium azide 1.0 mM was present in all buffers.

Preparation of cytosols. The specimens, 40-400 mg, were weighed, thawed and homogenized in 10-100 volumes of ice cold TEM buffer with an all glass Potter-Elvehjem homogenizer. Care was taken to avoid elevation of temperature. After homogenization adequate volumes (3 × 250 µl) were immediately removed for DNA determination (6). The remaining part was centrifuged at 20,000 × g for 10 minutes. The supernatant was then divided into 4 portions: 1) 100 µl for a preliminary protein estimation (ODU 280-310; one ODU = approximately 0.7 mg protein/ml (5)); 2) 100 µl for protein determination according to Lowry (7); 3) 300 µl for ER-

Key Words: Estradiol and progesterone receptor assay, breast cancer.

analysis and 4) 1.8 ml for PgR measurement. The pellet was used for a comparative DNA determination. Protein concentration was also measured in dextran-coated charcoal treated cytosol (see below). Usually nine samples were prepared on each occasion. The different steps in the analysis are summarized in Fig. 1.

Isoelectric focusing was performed on conventional polyacrylamide gels (Ampholine[®] PAG-plates, pH 3.5-9.5, LKB-Produkter AB, Bromma, Sweden) in a Multiphor instrument (LKB).

Counting. Radioactivity was counted in 5 ml Instagel[®] (Packard Instruments) in a liquid scintillation counter (Beckman LS-7000). Efficiency was determined with the external standard technique.

Estradiol receptor measurement. ER concentration was estimated with isoelectric focusing essentially as described by Wrangé *et al* (5) with slight modifications: the 300 µl portion of the supernatant was incubated with ³H-estradiol (final concentration 5 nM) for 1 hour at 0°C. CaCl₂ (final concentration 2 mM) and 2.5 mg trypsin per ODU₂₈₀₋₃₁₀ in TE buffer were then added. After 30 minutes at 10°C the samples were kept at 0°C. To remove excess estradiol, 100 µl DCC were added [dextran: charcoal 1:10 (w/v) in TE buffer, final concentration of charcoal 0.3% (w/v)]. The mixture was shaken for approximately 10 seconds and within one minute applied for centrifugation at 3,000 × g for 10 minutes. 100 µl of the supernatant were removed for protein measurement (7). The protein value was used for comparison with cytosol not DCC-treated. 150 µl were used for isoelectric focusing. After prefocusing for 30 minutes the samples were run for 1.5-2 hours. Ferritin was used as a control. A 2.5 cm wide rectangular area of the gel from the running field was removed and cut into 3 mm slices directly into the scintillation mixture. After extraction for 1 hour at 50°C radioactivity was measured. A peak of radioactivity representing specific binding appeared at the pH 6.3-6.4 region.

Progesterone receptor measurement. To 1.8 ml supernatant 0.2 ml glycerol was added. 100 µl aliquots were mixed with 50 µl of radioactive ligand (³H-R 5020 dissolved in TEK buffer). Five different concentrations of ligand (final concentration 0.2-4.0 nM) were used. For the determination of unspecific binding, 100 µl samples of cytosol were incubated with the strongest radioactive ligand concentration plus a hundred times excess of unlabeled ligand. After two hours incubation at 0°C, 20 µl DCC in TEK buffer were added [dextran: charcoal 1:10, final concentration of charcoal 0.3% (w/v)]. Absorption was carried out for 10 minutes with occasional shaking, followed by centrifugation at 3,000 × g for 10 minutes and counting of radioactivity. Total concentration of labeled ligand was counted in a mixture of 100 µl of cytosol + 50 µl ligand (4 nM) in TEK buffer + 20 µl of TEK buffer. The concentration of PgR in the cytosol and the K_d of receptor-ligand complex were calculated according to Scatchard (8).

Parameters of reference. ER and PgR concentrations in the tissue samples were calculated as femtomoles specifically bound ligand per mg tissue wet weight, per mg cytosol protein (before and after treatment with charcoal), or per mg DNA of the homogenate and residual pellet after centrifugation of the homogenate.

Control experiments. During the preparation of the cytosol, the influence of mercaptoethanol or dithiothreitol was tested, as was also the presence or absence of glycerol or radioactive estradiol. Centrifugation of homogenate was tested at 100,000 × g for 1 hour or 20,000 × g for 1 hour or 10 minutes. Measurement of ER concentration was made after absorption of excess ligand with two different charcoal concentrations [1.0 or 0.3% (w/v)] for two different periods of time (10 or 1 minute). In the routine measurement of PgR, excess R 5020 was removed with 0.3% (w/v) charcoal for 30 or 10 minutes or for the shortest time possible (1-2 minutes).

Results

Control experiments. The addition of mercaptoethanol to the buffer did not influence the results of ER measure-

ments (8 experiments) and the exchange of dithiothreitol to mercaptoethanol did not affect the PgR values (6 experiments). Addition of glycerol (PgR assay) or ³H-estradiol (ER assay) to the buffer before homogenization or to the samples after centrifugation did not alter the recovery of receptor (11 and 8 experiments respectively); a negative effect of glycerol on the ER measurements could not be excluded (3 experiments).

Centrifugation. Homogenates were centrifuged at 100,000 × g for 1 hour or at 20,000 × g for 1 hour or 10 minutes. No difference of PgR recovery was observed after centrifugation for 60 or 10 minutes at 20,000 × g (8 experiments). The importance of the lower g-value on ER and PgR measurements is indicated in Fig. 2. As shown in this figure, centrifugation at 20,000 × g resulted in an obvious increase in recovery of ER and PgR, the mean increase being 34% (ER) and 19% (PgR).

Treatment with dextrancoated charcoal. For the absorption of excess ligand in the measurement of ER, two different concentrations of charcoal [1.0 or 0.3% final concentration (w/v)] for two different periods of time (10 minutes or approximately 1 minute) were studied. The results show that the highest ER values were obtained when low concentration of charcoal was used for a short period of time (Fig. 3). Comparison between concentration and time shows that time is the most important factor for the receptor recovery. A similar experiment was made in the PgR measurements. The 0.3% charcoal concentration was tested for 30 or 10 minutes, or for the shortest time possible (1-2 minutes). The 10 minute period seemed to be optimal under the present conditions (6 experiments).

Sensitivity of ER assay and recovery of ER. Isoelectric focusing of ER makes possible the measurement of 1.0 fmol specifically bound radioactive estradiol per ml cytosol. With the procedure used in the present study, a distinct peak of radioactive ligand at the appropriate pH of the PAG plates was found in 414 (95%) out of 434 consecutive breast cancer samples. With the same criteria, ER was detected in 457 (75%) out of 607 samples in a previous series of samples analyzed before the modification of the method. The same pattern was obtained when this unselected tumour material was compared with specimens from stage II breast cancer. The concentration of ER increased in the unselected material, the median value being 1.5 fmol/mg wet weight. This should be compared with a median value of 0.6 in previous analyses. Differences were also observed when ER concentration was expressed on a protein or DNA basis. A reevaluation was then made of 438 ER analyses. In this series ER could not be quantitated in 26 (6%) patients, although in 19 (4%) of these patients presence of ER could not be excluded as a subtle increase of radioactive ligand was found at the characteristic isoelectric point of the estradiol receptor. These

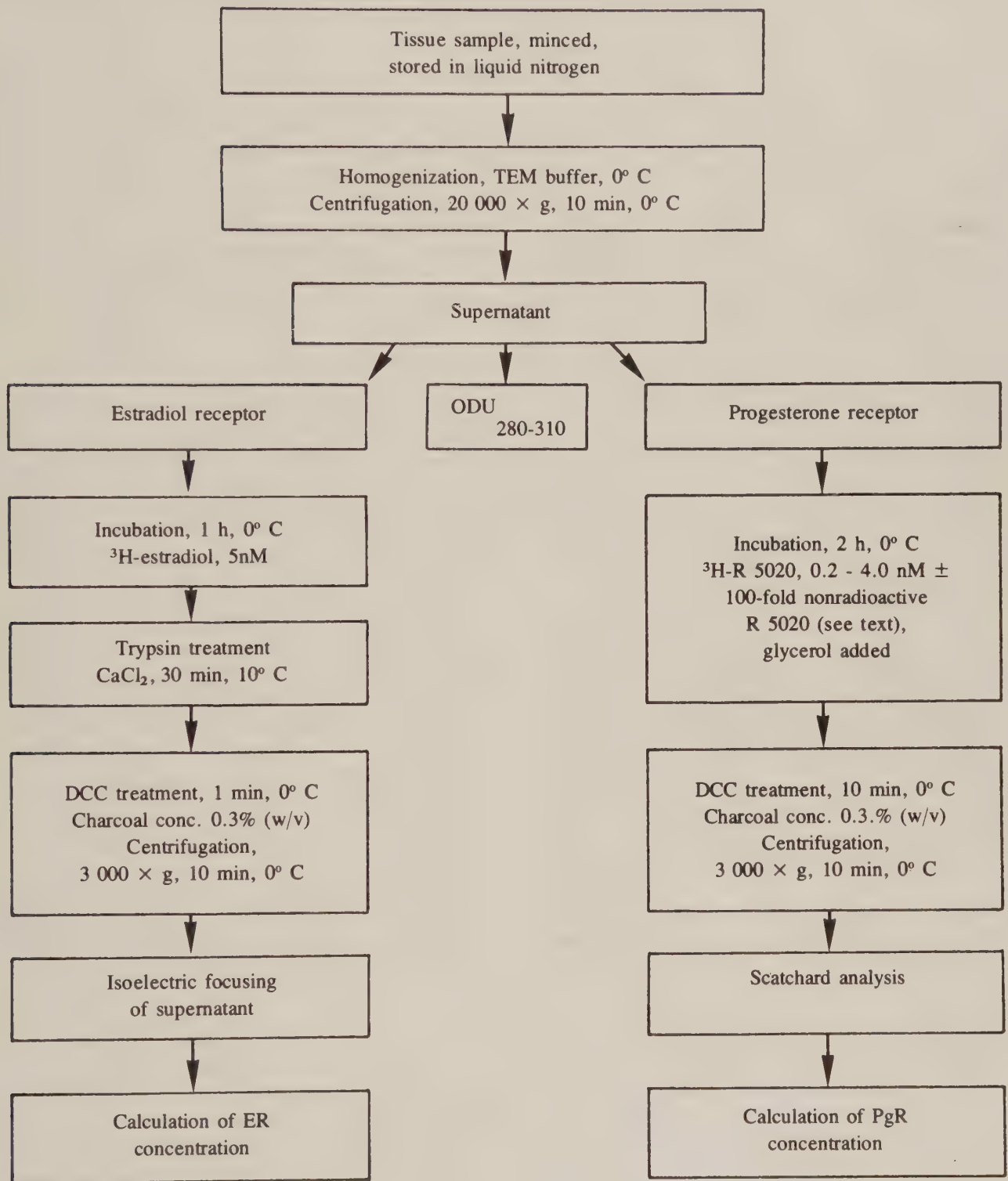


Fig. 1. Simplified scheme of assay procedure for routine determination of ER and PgR in cytosol. Details on protein and DNA assays are not shown.

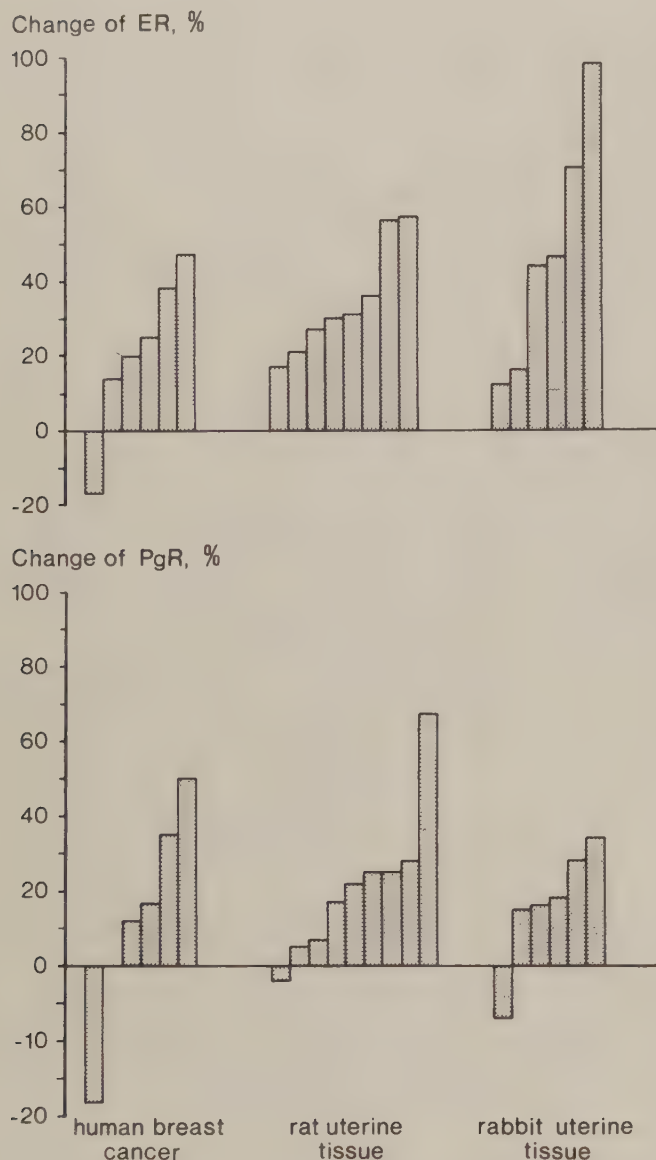


Fig. 2. Comparison of ER and PgR recovery in cytosol samples centrifuged at 20,000 $\times$ g or 100,000 $\times$ g. Each bar represents a comparison between two cytosol samples from the same tissue homogenate.

samples might represent so-called false ER negative tumours and if taken into consideration, 431 (98%) of a total 438 patients presented ER. A similar reevaluation of ER records previously analyzed showed that ER might have been present in 86% of the tumours.

The obvious increase of ER recovery was subject to two control experiments: (a) ER in 8 breast cancer specimens were simultaneously measured with the present and the previously used routine technique. The concentration of ER ranged between 0.02-21 fmol/mg tissue wet weight. The recorded increase of ER recovery was 30-260% (mean

90%). (b) A similar experiment was made in 4 rabbit and 4 rat uteri. Concentration of ER ranged between 2.34-15.9 fmol/mg tissue and the increase of recovery was 29-89% (mean 48%).

PgR assay. With the sensitivity of the multiple point assay (5-10 fmol specifically bound R 5020/ml cytosol), 210 (59%) out of 355 evaluable consecutive samples were found to contain PgR. In a previous consecutive sample series, analyzed with a slightly different method, PgR was detected in 218 (64%) out of 339 tumour samples. Median concentration of receptor calculated on a wet weight basis increased 30% with the present technique. Similar differences were found when receptor concentrations were calculated on a protein or DNA basis.

Discussion

In clinical practice information from ER measurements in breast tumour material may be incomplete due to the presence of low receptor concentrations analyzed with methods of low sensitivity in small amounts of tissue. The information may additionally include PgR measurements when possible (3). Representative tumour material may be difficult to obtain, since breast neoplasms often show considerable histologic heterogeneity (9, 10, 11).

Measurement of ER with isoelectric focusing (5) offers several advantages. The sensitivity of the method is comparatively high and in typical experiments, focusing of the specific estradiol binding protein is expressed as a distinct peak of radioactivity in the appropriate region of the pH gradient. The equipment used is easy to handle, is commercially available and not expensive. The method has been routinely used in our laboratory for measuring ER in breast tumour material. It should be noted that even minor changes in certain steps of the ER assay proved to be of unexpectedly high influence on the receptor recovery. This was true for the choice of g-value at centrifugation and even more for the treatment with DCC for the removal of excess ligand. As far as the DCC treatment was concerned incubation time was more important than the charcoal concentration. It seems that the short period of incubation (1 minute) with DCC was sufficient to remove adequate amounts of excess ligand without significantly interfering with the analysis. As for the PgR measurement the situation was slightly different. Here, a 10 minute period of exposure to charcoal proved to be most favorable. At longer exposure time the amount of measured receptor was decreased probably due to removal of both specifically and unspecifically bound ligand together with the hormone receptor. The short period (1-2 minutes) tested did not efficiently remove foremost unbound and not specifically bound ligand, resulting in less distinct curves. These experiments, together with those of others (12), show that incubation with DCC in both ER and PgR assays must be

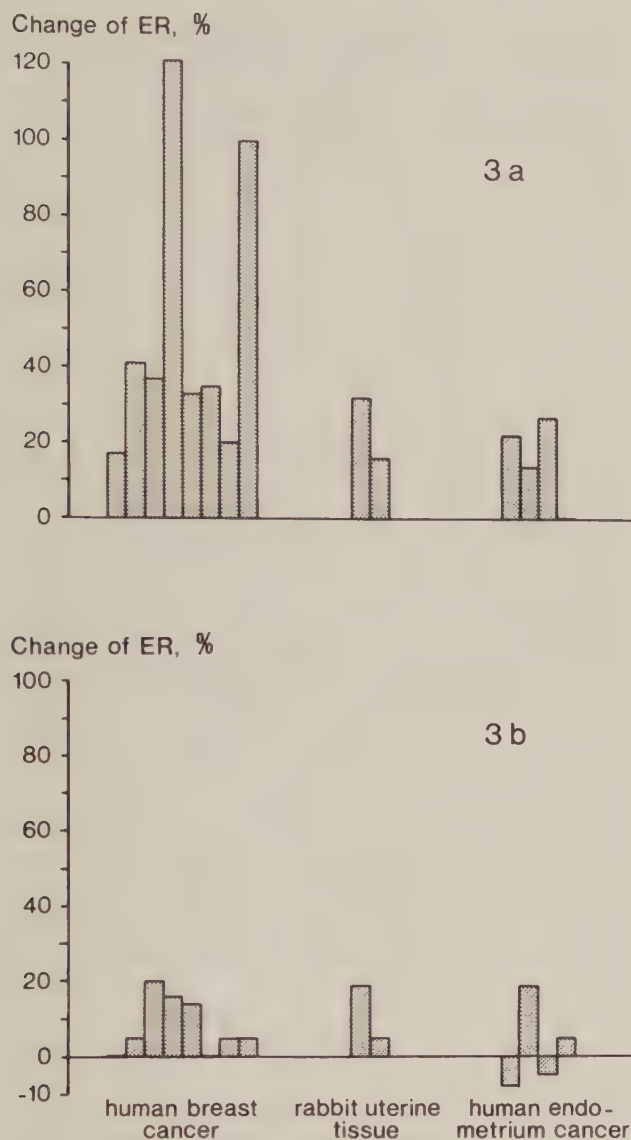


Fig. 3. ER and PgR recovery in cytosol samples at different conditions of incubation with dextran-coated charcoal (DCC). Each bar represents a comparison between two cytosol samples from the same tissue homogenate. (a) Effect of time: Comparison of ER recovery after one or 10 minute exposure to DCC. (b) Effect of DCC concentration: Comparison of ER recovery with 0.3 or 1.0% DCC (w/v).

performed under precisely standardized conditions.

After necessary control experiments it has been possible to combine the isoelectric focusing of ER with multiple point assay for PgR in the same cytosol obtained from small pieces of tissue. Inaccuracies due to tumour heterogeneity are partly avoided and the concentration of the two receptors may be expressed on the same comparable basis. When "false negative" samples (indicating presence of ER not possible to quantitate) were taken into consideration, very few tumours were strictly devoid of ER. This

might be explained simply by errors in sampling, inadequate conditions during transportation or technical difficulties. It should also be mentioned that many of the "false negative" samples were received thawed. Blocking of the receptor by endogenous hormone (13) offers further explanation of low ER in some tumours. This may be one of the reasons for the results obtained in a series of samples obtained from 44 pre- and 78 post-menopausal patients with stage II disease and analyzed with the present method. In this series ER was recorded in 40 (91%) and 76 (97%) samples respectively.

The increased concentrations of PgR recorded in this series and the reduced percentage of PgR containing tumours compared with previous results would seem contradictory. One reasonable explanation may be the fact that in the present method both ER and PgR are estimated in tissue quantities which previously permitted analysis of ER only. Under these conditions the amount of receptor may be so small as to escape detection in the interpretation of the saturation curves. A more sensitive method for the detection of PgR would, therefore, be desirable, and again, isoelectric focusing would present distinct advantages. Measurement of PgR with this procedure has been reported (14) and has been tested in our laboratory. In our hands the technique described resulted, however, in much lower recovery than that obtained with the DCC technique. An explanation for these contradictory results has not yet been found.

Cellular heterogeneity of most breast tumours (9, 10, 11) would seem to be a reasonable basis for the explanation of differences in receptor synthesis and degradation between different tumour cells and may be expressed biologically by significant differences in receptor concentration. These intricate matters cannot be resolved by present day methods of receptor analysis and point to the importance of developing means for the correction of receptor concentration with regard to tissue and cellular heterogeneity.

The observations made in the present work should be extended to other hormonally responsive human tumours as well. The present results may have implications on the clinical value of tumour ER status, since in this context it is shown that strictly hormonally non-responsive tumours are a rarity. On the other hand, the present results agree with the observation that a certain percentage of the so called ER negative tumours respond to hormonal therapy.

Furthermore, considering the heterogenous nature of most tumours, it seems difficult to identify a specific level of receptor concentration at which hormonal therapy would be of value in one single patient, since the fraction of high ER/hormonally responsive cells under treatment may be small. According to this notion, a varying but progressing fraction of hormonally responsive cancer cells may be present in nearly all patients with disseminated disease. This

information alone would invoke hormonal therapy single or in combination with other therapeutic regimens already from the start of a systemic treatment.

Acknowledgements

The present investigation was in part supported by grants from the Swedish Cancer Society, John and Augusta Persson's Foundation, and grants from the Medical Faculty, University of Lund.

References

- 1 Charnes GC and McGuire WL: Methods for analyzing steroid receptors in human breast cancer. In *Breast Cancer 3, Advances in Research and Treatment*. Plenum, New York, 1979, pp 149-197.
- 2 McGuire WL, Pearson OH and Segaloff A: Predicting hormone responsiveness in human breast cancer. In *Estrogen Receptors in Breast Cancer*. McGuire *et al* (eds), Raven Press, 1975, pp 17-30.
- 3 McGuire WL: An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. In *Progress in Cancer Research and Therapy*, Vol. 14, Hormones and Cancer. Raven Press, N.Y., 1980, pp 337-343.
- 4 Steroid receptors in breast cancer. NIH Consensus Development Conference Summary, Volume 2, No 6, 1979, pp 1-3.
- 5 Wrangé Ö, Nordenskjöld B and Gustafsson J-Å: Cytosol estradiol receptor in human mammary carcinoma: An assay based on isoelectric focusing in polyacrylamide gel. *Anal Biochem* 85:461-475, 1978.
- 6 Burton K: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 62:315-323, 1956.
- 7 Lowry OH, Rosebrough Nira J, Farr L and Randall Rose J: Protein measurement with the folin phenol reagent. *J Biol Chem* 193:265-275, 1951.
- 8 Scatchard G: The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 51:660-672, 1949.
- 9 Fisher ER: Pathology of breast cancer. In *"Breast Cancer Advances in Research and Treatment"*. Vol. 1: "Current Approaches to Therapy". Plenum, NY, 1977, pp 43-123.
- 10 Linell F, Ljungberg O and Andersson I: Breast carcinoma, aspects of early stages, progression and related problems. *Acta Pathol Microbiol Scand*, Section A, 1980, Supplement No. 272.
- 11 Heppner G: The challenge of tumor heterogeneity. In *Comments on Research in Breast Disease*, Vol. 1. Bulbrook RD (ed.). Alan R Liss, Inc., NY, 1979, pp 177-191.
- 12 Ralet P and Brombacher PJ: The use of coated charcoal in the determination of oestrogen receptor activity. *Eur J Nucl Med* 6: 159-162, 1981.
- 13 McGuire WL: Physiological principles underlying endocrine therapy of breast cancer. In McGuire WL (ed.): *"Breast Cancer Advances in Research and Treatment"*, Vol. 1: "Current Approaches to Therapy". Plenum, NY, 1977, pp 217-262.
- 14 Wrangé Ö, Ramberg I, Humla S, Gustafsson SA, Skoog L, Nordenskjöld B and Gustafsson J-Å: Estrogen and progestin receptor analysis in human breast cancer by isoelectric focusing in slabs of polyacrylamide gel. 1981. To be published in *Recent Results Cancer Res*.

Received April 28, 1982

A Comparison of Two Steroid Receptor Assays in Breast Cancer: Dextran Coated Charcoal and Isoelectric Focusing

MÅRTEN FERNÖ, ÅKE BORG and ALLAN NORGREN

Department of Oncology, University Hospital, S-221 85 Lund, Sweden

Abstract. *The multiple point dextran coated charcoal technique with Scatchard analysis (DCC) and isoelectric focusing on polyacrylamide gels (IF) were compared with regard to steroid receptor recovery, amount of tissue necessary, and expenses of assay. Quantitative correlations showed that when using DCC as compared to IF, estradiol receptor (ER) recovery increased two and progesterone receptor (PgR) recovery increased four times. In spite of lower recovery, IF may be a good alternative to DCC for routine ER assay since a small amount of tissue is required and the IF assay is easy to evaluate. The two assays were approximately equal in expense.*

One commonly used assay for cytosol estradiol and progesterone receptor is the multiple point dextran coated charcoal method (DCC). Calculations of receptor concentrations and affinity constants are made by Scatchard analysis (1, 2). One disadvantage of this method is the necessity of relatively large amounts of tissue for the preparation of a cytosol of sufficient volume and protein concentration. In addition, low concentration of receptor may cause difficulties in the interpretation of the saturation curves. Single point DCC assays can be performed with cytosol from a smaller amount of tissue, but these are less reliable than multiple point assays.

Isoelectric focusing (IF) is a recent technique for the identification and measurement of ER and PgR in the presence of a single concentration of labeled ligand (3, 4, 5). Here, receptor identification relies on its focusing at the specific isoelectric point in a continuous pH gradient on columns (3) or on polyacrylamide gels (4, 5).

A comparison was made between ER concentrations measured by the IF technique and by sucrose gradient centrifugation (4). IF was found to be a specific method for receptor identification, and receptor recovery was higher than with sucrose gradient centrifugation. In a comparison of the IF and DCC method, presence or absence of ER agreed in all but one of the 32 cytosols analyzed (7). ER negativity was defined as less than 5 fmol specifically

bound estradiol/mg total cytosol protein. It was noted, however, that the IF method measured significantly lower ER concentrations than the DCC technique. Isoelectric focusing of PgR has been reported (5) with a good correlation with sucrose gradient centrifugation.

With the modified IF and DCC techniques, as used routinely in our laboratory for the assay of ER and PgR in breast cancer tissue, ER was detected in more than 90% and PgR in 50-60% of unselected consecutive biopsies (6). In the present work, experiments have been designed in order to compare IF with DCC technique for the measurement of both ER and PgR in biopsy material from breast cancer.

Materials and Methods

Tissue: Biopsy material from human breast cancer was obtained at operation and was freed from fat, blood clot, etc., minced and then kept at either -70 °C or in liquid nitrogen until analysis. In control experiments (see below), material was obtained from mature rat and rabbit uteri.

Analytical procedures

Chemicals: All chemicals were of analytical grade.

Ligands: ³H-estradiol (85-115 Ci/mmol), ³H-promegestone (R 5020, 81-87 Ci/mmol) and nonradioactive promegestone were purchased from New England Nuclear, Boston, MA, USA. Nonradioactive estradiol was donated by LEO Pharmaceutical Company, Helsingborg, Sweden.

Buffer solutions: TEM (Tris-HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM, pH 7.4); TEK (Tris-HCl 10 mM, EDTA 1.0 mM, KCl 50 mM, pH 7.4); TE (Tris-HCl 10 mM, EDTA 1.5 mM, pH 7.4). Sodium azide 1.0 mM was present in all buffers.

Preparation of cytosol: ER and PgR have been measured in different tissue samples. Tissue material was homogenized in 10-20 volumes of TEM buffer at 0° C with an all glass Potter-Elvehjem homogenizer. For the measurement of PgR the buffer also contained glycerol, 10% (v/v) (final concentration when not otherwise stated). The homogenate was centrifuged at 20,000 × g for 10 minutes at 0° C, whereupon the supernatant was divided into two portions: 1/2.0 ml for DCC, 2/300 µl for IF. 100 µl (ER only) were removed for a preliminary protein estimation (ODU₂₈₀₋₃₁₀).

Dextran coated charcoal technique: Aliquots of supernatant were mixed with five different concentrations, 0.1-4.0 nM, of ³H-estradiol dissolved in TEM buffer or ³H-R 5020 (PgR-assay) dissolved in TEK buffer. For the determination of unspecific binding, cytosol was incubated with the strongest radioactive ligand concentration plus a hundred times excess of

Key Words: Breast cancer, steroid receptors, dextran coated charcoal, isoelectric focusing.

unlabeled ligand. After 2 hours incubation, absorption was carried out with DCC [dextran: charcoal 0.03:0.3% (w/v) in TE buffer (ER) or TEK buffer (PgR)]. Ten minutes of DCC treatment with occasional shaking was followed by centrifugation at $3,000 \times g$ for 10 minutes. Total concentration of labeled ligand was measured in a mixture of cytosol + ligand (4 nM) + TE (ER) or TEK buffer (PgR) in the same proportions as in the DCC treated tubes. Counting of radioactivity was made in 5 ml Instagel^R (Packard Instruments) with the external standard technique. The concentration of receptor in the cytosol and the K_d of receptor-ligand complex were calculated with Scatchard analysis (1, 2).

Isoelectric focusing: The methods were essentially as those described by Wrangé *et al* (4, 5) with some modifications (6) according to the following brief description:

The supernatant was incubated with 5 nM ³H-estradiol or 20 nM ³H-R 5020 for 1 hour (ER) or 1.5 hour (PgR) at 0° C. In the ER-assay the samples were incubated at 10° C for 30 minutes with 2 mM CaCl₂ and 2.5 mg trypsin/ODU₂₈₀₋₃₁₀, dissolved in TE buffer. In order to remove excess ligand, DCC was added [0° C, dextran: charcoal 0.03:0.3% (w/v) in TE buffer]. The mixture was shaken for approximately 10 seconds and within 1 minute centrifuged at $3,000 \times g$ for 10 minutes. This supernatant was used for isoelectric focusing, on conventional polyacrylamide gels [Ampholine^R PAG-plates, pH 3.5-9.5, LKB-Produkter, Bromma, in Multiphor instrument (LKB)]. In PgR measurement, polyacrylamide gels containing 20% glycerol were used. The radioactivity was counted as described previously (6).

Control experiments: In order to study the reproducibility of the respective methods, 4-9 aliquots of the same rat or rabbit uterus cytosol were analyzed separately. This was done at different levels of receptor concentration.

Results

Estradiol receptor. Comparisons between IF and DCC assay methods were made in 46 cytosols. The results are listed in Table I in increasing order of ER concentration as measured with IF. In two of the cytosols with no evidence of ER analyzed with IF, ER could be detected with the DCC technique. 43 samples had distinct peaks of radioactivity at the isoelectric point of the receptor. All but one of these cytosols (exp. 8) also had ER assayed by the DCC method. The K_d -values were less than 10×10^{-10} M except in six measurements. The latter were evenly distributed over the different ER concentrations. Quotients between DCC and IF values were calculated in 42 cases, all in favour of the DCC technique. The quotients were 1.1-33, with a median value of 2.0, as shown in Table I. The higher quotients occurred mainly in the lower region of ER concentrations.

Table II shows the variation in the quantitation of ER at 4 (IF) or 3 (DCC) different concentration levels in rabbit uterus cytosols. With the exception of the lowest concentration, the deviation from the mean value was generally less than 10%.

The correlation coefficient was 0.99.

Progesterone receptor. 77 cytosols were analyzed for PgR with IF and DCC techniques. Twelve cytosol samples were

eliminated from this comparison due to technical problems or difficulties in the interpretation of results. Of the remaining 65, 22 were negative with both assay methods. 43 showed PgR activity either with one or both of the assays used, and are indicated in Table III in increasing order of PgR concentration measured with IF. Three IF negative samples contained PgR as assayed with DCC. Six other cytosols, although negative with DCC, showed peaks of PgR with IF.

The Scatchard analysis showed variable K_d values, 50% being more than 10×10^{-10} M. DCC/IF quotients were calculated in 37 cases, all except one (exp. 28) were in favour of the DCC method. With this exception, quotients between 1.6 and 14 were found; the median value being 3.9.

Compared with the reproducibility of ER measurements the corresponding study concerning PgR assays gave higher variations. Table IV shows that in rat or rabbit uteri, at receptor concentrations below 100 pM, the maximal variation from the mean value was about 20%. Above 100 pM the fluctuations were approximately 5%, with the exception of IF analysis at a PgR concentration of 290 pM (40%). It should be noted that the protein concentration in this sample was comparatively high.

The correlation coefficient for the 43 samples showing PgR activity either with one or both methods was 0.92.

Discussion

Determination of steroid receptor concentrations have attracted great interest since knowledge of receptor status gives information of hormonal dependency and prognosis in breast cancer. For routine purposes several factors are of importance for the choice of the receptor assay. It should be reliable, sensitive, and easy to evaluate. Furthermore, the amount of tissue necessary, time requirements, and expense of equipment are also of potential interest.

Two commonly used techniques for the measurement of ER and PgR are the dextran coated charcoal method with Scatchard analysis (DCC) and sucrose gradient centrifugation. A third assay using isoelectric focusing (IF) has been compared with these methods (4,5) and was found to be a specific technique for receptor measurement although receptor recovery was variable.

In accordance with others (7), ER recovery was reduced when IF was compared with DCC in the present study; which also showed that the discrepancy was even more pronounced for the assay of PgR. The explanation may be found in the extra analytical steps involved in the IF techniques. Trypsin digestion of the receptor (ER-assay) and the separation of different proteins by isoelectric focusing are steps not required in the DCC method. As far as trypsin treatment is concerned this step in the assay would rather be expected to increase receptor recovery (4, 8). Running of the receptor-³H-ligand complex into the ligand-

TABLE I. Concentration of ER (pM) in cytosol prepared from 46 primary mammary tumours. Aliquots of cytosol were analyzed with isoelectric focusing (IF) or multiple point dextran coated charcoal technique (DCC). The DCC/IF quotients are shown. For details, see Materials and Methods.

Exp.	IF	DCC	DCC/IF
1	0	0	
2	0	6.4	
3	0	11	
4	0.3	10	33
5	0.9	25	28
6	1.3	23	18
7	2.3	5.8	2.5
8	2.5	0	
9	2.8	14	5.0
10	3.3	31	9.4
11	3.9	7.6	1.9
12	4.3	31	7.2
13	4.9	21	4.3
14	7.0	18	2.6
15	7.4	26	3.5
16	8.8	19	2.2
17	9.4	14	1.5
18	10	110	11
19	12	76	6.3
20	13	31	2.4
21	13	33	2.5
22	14	29	2.1
23	15	41	2.7
24	15	19	1.3
25	22	49	2.2
26	29	71	2.4
27	32	55	1.7
28	32	71	2.2
29	34	69	2.0
30	38	70	1.8
31	43	63	1.5
32	46	59	1.3
33	56	110	2.0
34	57	89	1.6
35	59	120	2.0
36	110	180	1.6
37	120	180	1.5
38	130	230	1.8
39	160	270	1.7
40	260	430	1.7
41	260	460	1.8
42	340	460	1.4
43	350	450	1.3
44	380	530	1.4
45	810	1300	1.6
46	2800	3000	1.1

TABLE II. Variations of estradiol receptor concentrations (pM) between separate analyses of the same cytosol preparation of rabbit uterus. DCC = multiple point dextran coated charcoal method; IF = isoelectric focusing; n = number of samples.

Assay	n	Mean receptor conc.	Maximal deviation %	Mean deviation %
IF	7	1800	2.6	1.3
	8	930	8.8	3.8
	8	89	11	4.7
	8	4.2	65	23
DCC	9	2400	4.2	1.7
	9	410	3.4	1.6
	9	28	23	9.6

TABLE III. Concentration of PgR (pM) in cytosol prepared from 43 primary mammary tumours. In 22 additional tumours PgR was not detected. Aliquots of cytosol were analyzed with isoelectric focusing (IF) or multiple point DCC technique (DCC), respectively. DCC/IF quotients are given when possible. For details, see Materials and Methods.

Exp.	IF	DCC	DCC/IF
1	0	26	
2	0	68	
3	0	120	
4	1.4	0	
5	1.6	0	
6	2.1	0	
7	2.2	0	
8	2.9	0	
9	3.3	0	
10	4.1	54	13
11	5.2	72	14
12	7.2	33	4.6
13	8.8	91	10
14	9.0	20	2.2
15	14	110	7.9
16	17	58	3.4
17	19	140	7.4
18	20	110	5.5
19	22	36	1.6
20	23	53	2.3
21	24	80	3.3
22	27	390	14
23	32	200	6.2
24	50	220	4.4
25	56	190	3.4
26	67	240	3.6
27	68	280	4.1
28	77	950	12
29	78	400	5.1
30	83	320	3.9
31	100	230	2.3
32	110	190	1.7
33	120	600	5.0
34	130	420	3.2
35	180	1400	7.8
36	220	860	3.9
37	260	1000	3.8
38	280	1500	5.4
39	450	2800	6.2
40	510	1800	3.5
41	640	2000	3.1
42	1200	4900	4.1
43	1600	3500	2.2

TABLE IV. Variations of progesterone receptor concentrations (pM) between separate analyses of the same cytosol preparation of rat or rabbit uterus. DCC = multiple point dextran coated charcoal method; IF = isoelectric focusing; n = number of samples.

Assay	n	Mean receptor conc.	Maximal deviation %	Mean deviation %
IF	9	290	42	18
	9	150	5.8	2.9
	9	11	25	10
DCC	6	680	3.4	1.7
	4	140	4.5	2.3
	4	67	21	11
	4	29	17	9.1
	3	22	14	9.4
	4	9.8	19	9.7

free environment on the gel leads to a progressing alteration in the receptor ligand equilibrium. This may result in an increasing dissociation and a decreasing amount of measurable receptor. In such a situation the peaks of radioactivity would be expected to be less distinct, which was also a regular finding in the PgR assay. The latter finding may also be due to the fact that the PgR-R 5020 complex is less stable than the ER-estradiol complex. For that reason, attempts were made to increase the measurable concentration of receptor (ER and PgR) with the addition of sodium molybdate to the buffer, which has been claimed to increase receptor recovery (10). However, in preliminary experiments recovery of ER and PgR was not changed in breast cancer cytosols. On the other hand, PgR recovery was increased when rat and rabbit uteri cytosols were used (data not shown).

The influence of decreasing pH gradient on the receptor molecule and the aggregation and precipitation of receptor at certain protein concentrations, are further possible explanations for the discrepancy between IF and DCC. In this respect the focusing of different proteins other than receptor may present rather intricate problems; factors which do not seem to have been studied. The factors mentioned above can, at least in part, explain the decreased receptor recovery using IF.

The difference in receptor concentration measured by DCC and IF technique cannot be explained by variations inherent in the methods used, since the control experiments showed satisfactory reproducibility. It should be emphasized that the experiments were made with rat and rabbit uterus cytosols in order to obtain enough tissue material. On the other hand adequate amounts of breast cancer tissue were obtained for similar control experiments with IF only. The results of these experiments were similar to those obtained with rat and rabbit cytosols. Nine separate samples were analyzed for ER and PgR from the same breast cancer cytosol. Mean deviation of ER concentration was 4.4% at high ER level. Corresponding value for nine PgR measurements was 27%.

One distinct advantage with IF compared to DCC is the smaller amount of tissue necessary for measurement, as illustrated by studies of ER in fine needle aspirates from breast tumours (9). This implies that due to dilution of receptor, samples with low receptor concentration may be negative with DCC but positive when using IF. Furthermore, the assays are easier to evaluate with IF at low receptor concentrations.

By the isoelectric focusing procedure ER and PgR are separated from sex hormone binding globulin (SHBG) and other steroid binding proteins (specific and unspecific). These proteins may cause problems in DCC technique influencing K_d-values and recovery. For that reason the effects of excess 5 α -dihydrotestosterone, by means of its binding to SHBG, on the recovery of receptor were studied with DCC. No significant change in K_d values or in recovery explaining the great discrepancy between IF and DCC

was shown in five breast cancer samples (data not shown).

In experiments made previously, a hundred times excess unlabeled estradiol eliminated peak of radioactivity at isoelectric focusing of ER. In DCC experiments unspecific binding was assessed mostly with the addition of hundred times excess unlabeled estradiol, but in additional control experiments no significant change of receptor values was found with diethylstilbestrol as unlabeled ligand. Thus the choice of nonradioactive ligand in the DCC method cannot explain the difference between IF and DCC assay.

As far as the time requirement and expenses of analysis are concerned the DCC and IF methods are comparable.

In conclusion, with the exception of receptor recovery, IF offers an assay comparable with DCC and is in other respects an even better choice than DCC for the routine measurement of ER in human breast cancer cytosols. As far as PgR is concerned the much lower recovery obtained by IF is unsatisfactory, meaning that DCC as compared to IF is a better method for the measurement of PgR.

Acknowledgements

The present investigation was supported in part by grants from the Swedish Cancer Society, John and Augusta Person's Foundation, and grants from the Medical Faculty, University of Lund.

References

- Scatchard G: The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 51:660-672, 1949.
- Chamness GC and McGuire WL: Scatchard plots: common errors in correction and interpretation. *Steroids* 26(4):538-542, 1975.
- Wrange Ö, Nordenskjöld B, Silfverswärd C, Granberg PO, and Gustafsson J-A: Isoelectric focusing of estradiol receptor protein from human mammary carcinoma - a comparison to sucrose gradient analysis. *European Journal of Cancer* 12:695-700, 1976.
- Wrange Ö, Nordenskjöld B, and Gustafsson J-A: Cytosol estradiol receptor in human mammary carcinoma: an assay based on isoelectric focusing in polyacrylamide gel. *Analytical Biochemistry* 85:461-475, 1978.
- Wrange Ö, Humla S, Ramberg I, Gustafsson S, Skoog L, Nordenskjöld B, and Gustafsson J-A: Progesterone-receptor analysis in human breast cancer cytosol by isoelectric focusing in slabs of polyacrylamide gel. *Journal of Steroid Biochemistry* 14:141-148, 1981.
- Norgren A, Borg A, Fernö M, Johansson U, Lindahl B, and Tsiobanelis K: Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer. *Anticancer Research* 2:315-320, 1982.
- Lloyd EJ, Barnes DM, and Skinner LG: Isoelectric focusing of estradiol receptor protein from human mammary carcinoma - a comparison with dextran coated charcoal assay. *Journal of Steroid Biochemistry* 16:239-244, 1982.
- Pettersson K, Vanharanta R, Söderhom J, Punnonen R, and Lövgren T: Increase in the estrogen binding capacity of breast cancer cytosols following limited proteolysis with trypsin. *Journal of Steroid Biochemistry* 16:369-372, 1982.
- Silfverwärd C and Humla S: Estrogen receptor analysis on needle aspirates from human mammary carcinoma. *Acta Cytologica* 24:54-57, 1980.
- Anderson KM, Phelan J, Marogil M, Hendrickson C, and Economou S: Sodium molybdate increases the amount of progesterone and estrogen receptor detected in certain human breast cancer cytosols. *Steroids* 35(3):273-280, 1980.

Received January 5, 1983

**OBSERVATIONS ON WET WEIGHT, PROTEIN AND DNA
AS REFERENCE FOR STEROID RECEPTORS
IN MALIGNANT MAMMARY TUMOURS**

Allan Norgren,¹ Mårten Fernö & Åke Borg

**Department of Oncology University Hospital,
S-221 85 LUND, Sweden**

**¹Department of Clinical Physiology, University Hospital
S-751 85 UPPSALA, Sweden**

ABSTRACT

Estradiol and progesterone receptors together with DNA and soluble protein per unit wet weight of 235 breast cancer samples were recorded. Receptor concentration values per wet weight showed the greatest range of variation, and receptors expressed per unit wet weight, DNA or protein showed good correlations ($r_s > 0.90$). However, in selected cases different information as to level of receptor concentration may be obtained depending on reference parameter used. An overrepresentation was found of tumours with low receptor concentrations or receptors below the level of detection in a subgroup with a low DNA content, indicating the possibility of false low receptor values in this group. High ER and PgR contents were significantly related to high levels of tissue DNA. Low or high content of protein was not associated with low or high receptor levels. In this respect DNA is superior to wet weight and protein as reference standard for receptor concentration. In addition, DNA as reference offers the possibility of a rough correction for nonepithelial components of the tumours. A critical dilution of receptors as shown by low DNA content in some homogenates was not apparent, but cannot be excluded.

INTRODUCTION

Steroid receptor content is one of the novel means in the clinical evaluation of malignant breast tumours. It is generally accepted that patients with receptor positive tumours more often will respond to hormonal therapy than those with receptor negative tumours (1). However, several cases not following this pattern are frequently encountered in clinical practice. Among possible explanations may be breast cancer heterogeneity and methodological difficulties (2,3). Further, the correct way of expressing receptor content may be difficult as the malignant epithelial cells in breast cancer - of varying degrees of differentiation and probably also receptor content - are present in variable proportion together with normal epithelial cells, stromal tissue, fat, and in some tumours an infiltration of lymphocytes. In addition, small amounts of tissue available may at times be the cause of dilution of receptor beyond the possibility of detection.

There are three ways in common use to express receptor concentrations: on the basis of 1/ wet weight, 2/ protein or 3/ DNA. It seems that classification of tumours with regard to receptor content only to a minor extent is dependent on the reference parameter mentioned (4,5,6) in spite of the fact that the reference parameters are all influenced by the varying composition of the tumours. For protein the contamination of blood proteins to the biopsy material may also be of importance. When DNA is used as reference, receptor concentration reflects some kind of mean receptor content per tumour cell, provided that the contribution of DNA from other cells is negligible. Surprisingly, DNA is not often used as

the reference standard for receptors in breast tumour material in spite of the possibility to roughly correct for non-epithelial tumour content. Such a correction has been reported to be of importance (7).

In the present work, receptor concentrations were calculated per wet weight, soluble protein and tissue DNA of the same breast tumour biopsy. Soluble protein and DNA per wet weight of the tissue were also recorded. The aim was to study whether any of the reference parameters offered any advantage or disadvantage over the other ones.

MATERIAL AND METHODS

Tissue

Biopsy material obtained from 235 consecutive breast cancer patients were used for routine analysis of estrogen (ER) and progesterone receptors (PgR). Prior to analysis the tissue was freed from fat, blood clot etc., minced and then kept in liquid nitrogen for not more than two weeks.

Analytical procedures

Chemicals: All chemicals were of analytical grade.

Ligands: ^3H -estradiol (85-115 Ci/mmol) was purchased from Radiochemical Centre, Amersham England. ^3H -promegestone (^3H -R 5020, 81-87 Ci/mmol) and unlabelled R 5020 were obtained from New England Nuclear, Boston Ma. U.S.A. Non-radioactive estradiol was generously supplied by LEO Pharmaceutical Company, Helsingborg Sweden.

Steroid receptor determinations. Weighed tissue material was homogenized in approximately 20-30 volumes of buffer (Tris-HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM, pH 7.4) at 0°C with an all-glass Potter-Elvehjem homogenizer. Aliquots were removed for DNA determination after thorough mixing (see below). The remaining part of the homogenate was centrifuged at $20,000 \times g$ for 10 minutes at 0°C . The supernatant was used for the determination of ER and PgR as described previously (8). The separation of specifically from non-specifically bound ^3H -ligand was for both receptor assays performed with dextran coated charcoal (DCC). ER was measured with isoelectric focusing showing a distinct peak of radioactivity at pH 6.3-6.5 (9,10). The concentration of PgR was assessed with the multiple point dextran coated charcoal technique and Scatchard analysis. The level of sensitivity for ER and PgR was 1.0 and 10 pM, respectively (8). Receptor concentrations were expressed as fmol specifically bound ^3H -estradiol or ^3H -R 5020 (PgR) per unit wet weight, DNA and protein.

Protein determination. To aliquots, collected after treatment with DCC (8), trichloroacetic acid (TCA, 7.5% final concentration) was added. After centrifugation the pellet was resuspended and the protein concentration was measured according to Lowry *et al.* (11), with lyophilized bovine serum albumin as standard. Soluble protein per wet weight of the tumour specimen was recorded.

DNA determination. Homogenate samples were treated two times with TCA (10%; 0°C) and then extracted with TCA (5%; 90°C (12)). The DNA content was then determined according to the diphenylamine reaction (13) with calf thymus (sodium salt, Sigma) as DNA standard. DNA content per wet weight of tumour biopsy and per ml homogenate was calculated.

Statistics

Correlations were calculated with one-sided Spearman's rank correlation test. Evaluation of differences was made with χ^2 -analysis and Mann-Whitney's test; p-values less than 0.05 were considered statistically significant.

RESULTS

Receptor concentrations and profiles. ER was found in 216 of 235 and PgR in 102 of 217 tumour biopsies (92%; "ER positive"; 47%; "PgR positive"). In 18 samples PgR was not assayed or the assay was not possible to evaluate.

Mean values, standard deviations and ranges of receptor positive samples for the three reference parameters are shown in detail in Table 1. The distribution of receptor values on the basis of wet weight (ww) is given in Fig. 1, which shows a wide variation of both ER and PgR and an

obvious absence of PgR at the lower levels. The quotients between the highest and lowest ER and PgR concentrations were approximately 7,200 and 280, respectively. The receptor profiles are shown in detail in Table 2. From the table it can be seen that approximately half of the ER positive samples were PgR positive also and that one of the 19 ER negatives was of the ER negative/PgR positive type.

Table 1.
Mean ($\bar{x}$), standard deviation (SD) and range of concentration of steroid receptors in 216 ER positive and 102 PgR positive mammary tumours related to wet weight (ww) of the tissue, protein and tissue DNA.

Reference (mg)	ER (fmol)			PgR (fmol)		
	$\bar{x}$	SD	Range	$\bar{x}$	SD	Range
ww	3.6	7.8	0.01-72	7.7	11	0.20-56
Prot.	99	190	0.26-1,600	210	260	6.4-1,400
DNA	940	1,500	1.7-9,900	2,300	2,900	2.8-14,000

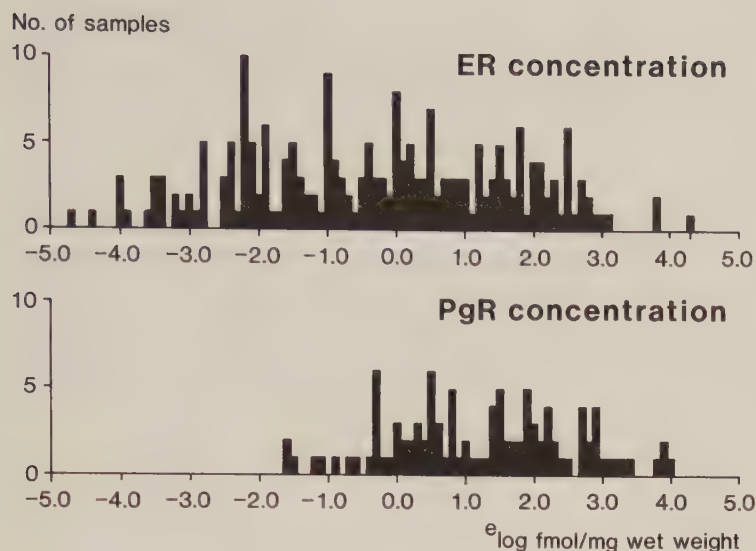


Figure 1.
Distribution of estradiol and progesterone receptor concentrations in receptor positive tumour biopsies expressed on the basis of wet weight. For details, see Results.

Table 2.
Receptor profile in 217 consecutive mammary carcinomas assayed with isoelectric focusing (ER) and the multiple point dextran coated charcoal technique with Scatchard analysis (PgR).

Profile	n	%
ER+/PgR+	101	46.5
ER+/PgR-	97	44.7
ER-/PgR+	1	0.5
ER-/PgR-	18	8.3

Receptor concentrations expressed per unit wet weight, protein and DNA. A rank correlation test was made for ER positive samples expressed per unit ww, protein and DNA. A strong positive correlation was found irrespective of the parameter used for comparison ($r_s > 0.96$). For all PgR positive tumours the corresponding figure was 0.91.

In clinical practice tumours are classified as either receptor positive (rich) or receptor negative (poor) on the basis of an arbitrarily chosen cut-off point. The question was raised whether the choice of reference in some cases would influence the classification and finally the choice of treatment of the patient. In the present study the cut-off point was set at 10 fmol ER/mg protein. Protein was chosen because of its frequent usage as reference standard. Ninetyfour (40%) tumours were found below the level chosen. With this number of biopsies below the cut-off point the corresponding value for ER/mg DNA or ww turned out to be 120 and 0.29 fmol, respectively. The number of tumours subjected to misranking due to the choice of parameter could then be estimated. Depending on the parameters compared, the number of misranked tumours were 16 (DNA-ww), 12 (protein-DNA) and 8 (protein-ww), respectively. For PgR a similar estimation of misranking of tumours was made when the cut-off point for PgR was set at 30 fmol PgR/mg protein, which resulted in 152 (70%) tumours falling below this level. The number of tumours subject to misranking were 8 (DNA-ww), 6 (protein-DNA) and 4 (protein-ww), respectively; cut-off points were 350 and 1.0 fmol per mg DNA and ww, respectively.

DNA and protein per unit wet weight were recorded and the results are shown in Fig. 2 and 3. For comparison with Fig. 1, DNA and protein per ww were for convenience indicated as $e^{\log}$ values. The receptor content shows by far the greatest variation, followed in order by DNA and protein. Mean $\pm$ standard deviation (SD) was for DNA 3.4 ± 1.9 mg/g ww ($n=235$) and for protein 35 ± 13 mg/g ww ($n=235$). A positive correlation ($r_s=0.60$) was calculated between DNA and protein per unit ww. Ratios between the highest and lowest values for DNA and protein were 61 (0.23 - 14 mg/g ww) and 13 (9.1 - 120 mg/g ww), respectively. For recollection, the ratio between the highest and lowest ER concentration per mg ww was 7,200 and 280 for PgR.

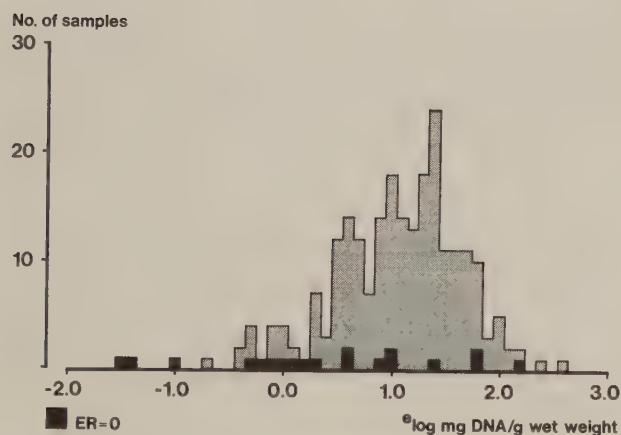


Figure 2.
Histogram showing content of DNA in mammary tumour biopsies. Mean $\pm$ SD = 1.1 ± 0.60 . Filled bars indicate ER negative assays.

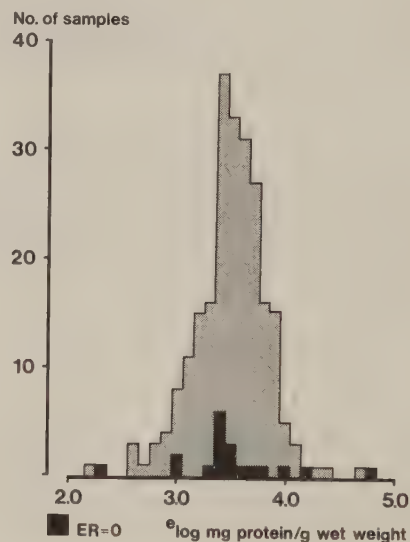


Figure 3.
Histogram showing content of cytosol protein per gram wet weight of mammary tumour biopsies. Mean $\pm$ SD = 3.5 ± 0.31 . Filled bars = ER negative assays.

ER in relation to DNA and protein content of the tumour.

In the present material ER was not detected in 19 tumours (8.1%). The mean DNA content for these 19 biopsies was 2.3 ± 2.3 mg/g ww, compared with 3.4 ± 1.9 for the 216 ER positive tumours. The difference was significant ($p < 0.005$). Among 29 samples with the lowest DNA content (< 1.5 mg/g ww; i.e. $< \text{mean} - 1 \text{ SD}$) 10 (34%) were ER negative. The results are indicated in the histograms of Fig. 2. The expected percentage if ER and DNA were independent would be 8.1 per cent ($P < 0.001$).

In order to examine the DNA content for samples with higher ER concentrations, a group representing the highest quartile of ER positive samples (> 3.7 fmol/mg ww) was chosen. The group included 54 tumour samples, in the following called ER rich. Their mean DNA content was 4.1 ± 1.6 mg/g ww compared with 3.1 ± 2.0 for samples with ER concentrations lower than or equal to 3.7 ($p < 0.001$). Fourteen (41%) of them were found in a subgroup

of 34 samples with the highest DNA content (>5.3 mg/g ww; i.e. $>\text{mean}+1$ SD). The expected percentage would have been 23 ($p<0.01$). The overall correlation between ER and DNA on the ww basis was 0.29 (r_s ; $p<0.001$).

The relation between low ER and protein content is shown in Fig 3. The pattern is different from that shown for tissue DNA content: mean protein was almost the same, 38 and 37 mg/g ww, for the ER negative and ER rich tumours. Three (14%) ER negative samples were found among the 21 with the lowest protein content (per unit ww; <22 mg/g; i.e. $<\text{mean}-1$ SD). The percentage to be expected if ER and protein were independent was 8.1 (NS).

Similarly, 3 (18%) of 17 samples with high protein content (>48 mg/g ww, i.e. $>\text{mean}+1$ SD) were ER rich. The expected value would have been 23 per cent (NS).

The r_s -value between ER and protein on the ww basis was 0.08 (NS).

PgR in relation to DNA and protein content of the tumour. PgR was not detected in 115 of 217 tumours. The mean DNA content in the PgR negative samples was 3.2 ± 2.0 mg/ww, to be compared with 3.5 ± 1.8 in the PgR positive samples (NS). However, in analogy with the low ER concentrations, PgR negatives were also overrepresented in the low DNA group. Thus, 22 (81%) PgR negatives were found in a group of 27 samples with low DNA content (<1.5 mg/g ww). The expected percentage if PgR and DNA were independent would be 53 ($p<0.005$).

DNA content of 34 PgR rich tumours (>6.7 fmol/mg ww), which represented the highest one third of the PgR positive samples, was 4.0 ± 2.2 mg/g ww compared with 3.2 ± 1.9 for samples with PgR concentrations less than 6.7 fmol/mg ww ($p<0.05$). Five of 31 samples (16%) with a DNA content higher than 1 SD from the mean value (>5.3 mg/g ww) were PgR rich, i.e. equal to the expected percentage.

The r_s -value between PgR and DNA on the ww basis was 0.18 ($p<0.005$).

Subgroups of tumours characterized by low or high protein content in combination with low or high PgR concentrations, respectively, could not be distinguished.

The r_s -value between PgR and protein on the ww basis was 0.02 (NS).

ER and PgR in relation to DNA concentration in homogenate. As low DNA content on ww basis was correlated with low or negative receptor concentrations, homogenate concentration of DNA was taken into consideration as an indicator of a potential source of error due to dilution of receptor. It was found that ER and PgR negative samples were overrepresented in the group with a low DNA concentration in the homogenate ($<36\mu\text{g}$ DNA/ml

homogenate; i.e. mean-1 SD): among 12 samples in this group, 5 (42%) were ER negative and 10 (91%) of 11 samples were PgR negative. The expected percentages were 8.1 ($p < 0.001$) and 53 ($p < 0.05$), respectively. The association between tissue content of DNA and homogenate DNA concentration was obvious ($r_s = 0.73$). By comparing the figures with those of the foregoing section, an undue dilution of receptor may have occurred in some few assays but was not apparant even if the material was extended to a total of 292 assays in this part of the study.

DISCUSSION

In the present tumour material, contents of steroid receptors, DNA and protein per wet weight were recorded. Receptor content was by far the most variable, followed by DNA and protein. Of the receptors, PgR varied less than ER with an absence of PgR corresponding to the lower region of the ER distribution. This may at least in part be explained by differences in methodology due to the favourable level of sensitivity of the isoelectric focusing procedure used for ER measurements. As a consequence some false PgR negative tumours may be present in this serie of assays.

The wide distribution of receptors, the comparatively narrow distribution of DNA and protein, and the positive correlation between DNA and protein ($r_s = 0.60$) may explain why receptor concentration expressed per unit wet weight (ww), DNA or protein showed a good correlation. For the present series the correlation coefficients using Spearman's rank correlation test were higher than 0.90 and it was almost the same irrespective of the reference parameter used. The positive correlation is in accordance with the findings of other investigators (4,5,6). In a large series of observations permitting statistical analysis, the different reference standards would seem, then, to have the same significance. In individual patients, as in the evaluation of breast disease in clinical practice, different information may be obtained depending on the reference parameter used.

ER negative samples were overrepresented in the subgroup of tumours with low DNA content. When including the lowest ER positive quartile (< 0.15 fmol/mg ww; $n = 56$), it was found that 21 (72%) of 29 low-DNA tumours had ER concentrations less than 0.15 fmol/mg ww. If ER and DNA were independent the expected percentage would have been 32 ($p < 0.001$). A similar overrepresentation of tumours with low PgR concentrations were also found in this group. It may be asked whether low or non-detectable receptor in low-DNA biopsies represent true or false low-receptor tumours, raising the important question of the predictive significance of receptor values at the low or boarderline concentrations. The crucial

characteristic of the biopsies in question was found to be low DNA content, indicating the possibility that receptor may have escaped detection. Undue dilution of receptor may have occurred in some few samples only, and an increased frequency of small tumour biopsies could not be found in the group. A step towards the elucidation of the complicated matter would therefore seem to be an evaluation of receptor concentrations on the basis of tissue DNA together with tissue content and homogenate concentration of DNA, fraction of tumour cells in the biopsy material or, perhaps more sophisticated, the recently developed immunocytochemical staining of receptor bearing cells of the tumours. A crude correction of the steroid receptor concentration would then be possible, similar to the procedure suggested previously by others (7). With the assay procedure used in this study the samples among which the lowest receptor concentration were found - and consequently at risk of being incorrectly interpreted - contained less than 1.5 mg of DNA per gram of tissue notably if the homogenate concentration of DNA was 40 $\mu\text{g/ml}$ or less.

In spite of the good correlation between receptor values expressed per unit wet weight, DNA or protein, information of DNA per unit wet weight or homogenate volume may help to identify erroneously low estimates or absence of steroid receptors. Comparison of receptor and DNA content with histopathology may be helpful to facilitate the identification of specific samples. For this reason DNA may be the reference parameter of choice for expression of steroid receptor concentrations. Protein content, although easy to measure, could not be used to indicate an increased probability of false low-receptor tumours in the assays made.

ACKNOWLEDGEMENTS

The present investigation was supported in part by grants from the Swedish Cancer Society, Swedish Medical Research Council, John and Augusta Person's Foundation and grants from the Medical Faculty, University of Lund, Sweden. The authors wish to express their gratitude to Ulla Johansson and Gunilla Sellberg for skilful technical assistance and to Bo Gullberg for statistical advice.

REFERENCES

1. McGuire WL: An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. In *Progress in Cancer Research and Therapy*, Vol. 14, Hormones and Cancer. Raven Press, N.Y., 1980, 337-343.
2. Fisher ER: Pathology of Breast Cancer. In *Breast Cancer 1, Advances in Research and Treatment* (McGuire WL, ed). N.Y., Plenum Publ. Corp., 1977, 43-123.
3. Chamnes GC and McGuire WL: Methods for analyzing steroid receptors in human breast cancer. In *Breast Cancer 3, Advances in Research and Treatment* (McGuire WL, ed). N.Y., Plenum Publ. Corp., 1979, 149-197.
4. Hähnel R and Twaddle E: Estradiol binding by human breast carcinoma cytosols: The influence of commonly used reference parameters on the results. *Eur J Cancer* 14, 1978, 125-131.
5. Allegra JC, Lippman ME, Thompson EB, Simon R, Barlock A, Green L, Huff KH, Do HMT, and Aitken SC: Distribution, frequency, and quantitative analysis of estrogen, progesterone, androgen, and glucocorticoid receptors in human breast cancer. *Cancer Res* 39, 1979, 1447-1454.
6. King RJB, Barnes DM, Hawkins RA, Leake RE, Maynard PV, Millis RM, and Roberts MM: Measurement of oestrogen receptors by five institutions on common tissue samples. In *Steroid Receptor Assays in Human Breast Tumours: Methodological and Clinical Aspects* (King RCB, Ed) Cardiff, Alpha Omega Publ Ltd, 1979. 7-15.
7. van Netten JP, Algard FT, Coy P, Carlyle SJ, Bridgen ML, Thornton KR, and To MP: Estrogen receptor assay in breast cancer microsamples. - Implications of per cent carcinoma estimation. *Cancer* 49, 1982, 2383-2388.
8. Norgren A, Borg Å, Fernö M, Johansson U, Lindahl B, and Tsiobanelis K: Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer. *Anticancer Res* 2, 1982, 315-320.
9. Wrangé Ö, Nordenskjöld B, and Gustafsson J-A: Cytosol estradiol receptor in human mammary carcinoma: An assay based on isoelectric focusing in polyacrylamide gel. *Anal Biochem* 85, 1978, 461-475.
10. Gustafsson J-A, Gustafsson SA, Nordenskjöld B, Okret S, Silverswärd C, and Wrangé Ö: Estradiol receptor analysis in human breast cancer tissue by isoelectric focusing in polyacrylamide gel. *Cancer Res* 38, 1978, 4225-4228.
11. Lowry OH, Rosebrough N, Farr L, and Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193, 1951, 265-275.
12. Schneider WC: Phosphorous compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J Biol Chem* 161, 1945, 293-303.
13. Burton K: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 62, 1956, 315-323.

**PROGNOSTIC SIGNIFICANCE
OF ESTROGEN AND PROGESTERONE RECEPTORS
IN STAGE II BREAST CANCER**

**Stefan Rydén,¹ Mårten Fernö,² Åke Borg,³ Larsolof Hafström,⁴
Torgil Möller⁵ & Allan Norgren⁶**

The Southern Swedish Breast Cancer Study Group

**From the Departments of Surgery (1, 4) and Oncology (2, 3, 5, 6)
University Hospital, S-221 85 LUND, Sweden**

**Present address: 4) Department of Surgery, Sahlgren Hospital,
Göteborg, Sweden**

**6) Department of Clinical Physiology,
University Hospital, Uppsala, Sweden**

**The full list of participating institutions and principal investigators
appears in the Appendix**

ABSTRACT

Estrogen (ER) and progesterone receptors (PgR) were evaluated in samples from primary tumors in 539 patients with stage II breast cancer (184 premenopausals and 355 postmenopausals) participating in a randomized multicenter controlled clinical trial on adjuvant therapy. The patients were followed according to a strict schedule with a median follow up of 39 months for premenopausal patients and 32 months for postmenopausal patients. All receptor assays were performed in one laboratory. ER was measured with isoelectric focusing and PgR with the multiple point dextran-coated charcoal method and Scatchard analysis. No correlation between receptor values and node involvement was observed.

At the chosen cut-off points of 10 fmol ER/mg protein and 30 fmol PgR/mg protein, no significant difference in recurrence free survival (RFS) existed between premenopausal patients with ER+ or PgR+ tumors and ER- or PgR- tumors, respectively. However, by changing the cut-off points to 40 or 50 fmol ER/mg protein and to 40 fmol PgR/mg protein or higher, respectively, a significant longer RFS were found for patients with receptor values above the cut-off points when compared with those with values below the cut-off points. Premenopausal patients with receptor positive (≥ 10 fmol ER/mg protein and ≥ 30 fmol PgR/mg protein, respectively) tumors had a significant longer survival than patients with receptor negative tumors. This was ascribed to a longer survival after recurrence.

In postmenopausal patients not treated with adjuvant endocrine therapy, no significant difference in RFS existed between patients with receptor positive and receptor negative tumors, irrespective of chosen cut-off point. Patients with ER+ tumors demonstrated, however, prolonged survival in comparison with patients with ER- tumors.

However, in postmenopausal patients treated with adjuvant tamoxifen, highly significant differences in both RFS and survival were observed when ER+ patients were compared with ER- patients or when PgR+ patients were compared with PgR- patients. No differences in survival after recurrence were observed among these patients.

The results suggest that for postmenopausal patients, ER and PgR values can not be used as independent predictors of prognosis, but are indicators of response to endocrine treatment, either as adjuvant or as treatment for metastatic disease. For premenopausal patients, high values of ER and PgR are associated with a longer RFS.

INTRODUCTION

The value of estrogen receptor (ER) analysis in the prediction of response to endocrine treatment in metastatic breast cancer is well established (1-3). The addition of progesterone receptor (PgR) measurement have further improved the accuracy of predicting the therapeutic response in advanced disease (4,5). Controversies still exist regarding hormone receptors as independent prognostic predictors in primary breast cancer. Several investigators have reported a better prognosis, in terms of a prolonged recurrence free survival (RFS), in patients with ER+ tumors (6-8) and similar findings have been reported regarding PgR+ tumors (9). Other studies have, however, failed to show any correlation between

RFS and the content of ER or PgR of the primary tumor (10-14). Correlations between ER or PgR and RFS in patients treated with adjuvant tamoxifen have been noted in two large trials (15,16). However, in another trial, ER- and ER+ patients responded equally well to adjuvant tamoxifen treatment (17). In a recent study, Howell et al. (18) reported a longer survival in patients with receptor positive tumors although no significant difference was found regarding RFS.

The purpose of the present study was to investigate if ER and/or PgR levels were related to RFS, overall survival and survival after relapse in patients with stage II breast cancer, and whether such a relation was correlated with the modality of adjuvant therapy.

MATERIAL AND METHODS

Patient material.

Five hundred and thirty-nine patients with stage II breast cancer were analyzed regarding the concentration of ER and PgR of the primary tumor. 184 patients were premenopausal and 355 were postmenopausal, i.e. more than five years after menopause. These patients were part of a prospective, multicenter, controlled clinical trial on adjuvant therapy of stage II breast cancer, which has been described in detail elsewhere (19). All patients were operated with modified radical mastectomy. Staging was based on the histo-pathological examination of the breast and axillary glands. Bone-scan and chest X-ray were negative regarding metastatic disease.

Since hormone receptor measurements were not prerequisites for entering the trial, the present study includes only that subset of patients in which ER and PgR analyses were performed in one, well-controlled, laboratory and in which tumor samples reached the laboratory properly frozen and in sufficient amount (n=539). Samples from patients on hormonal treatment were also excluded. Due to these criteria, 14% of the samples reaching the laboratory were excluded. No stratification according to receptor levels was performed.

Premenopausal patients were randomized to one of three postoperative treatment groups: radiotherapy alone, radiotherapy and cyclophosphamide, and cyclophosphamide alone. Radiotherapy was carried out according to a method described in detail elsewhere (20). Cyclophosphamide was administered p.o. in 12 four-week cycles with a daily dose of 130 mg/m^2 the first two weeks of each cycle.

Postmenopausal patients were randomized to one of the following postoperative treatments: radiotherapy alone, radiotherapy and tamoxifen, and tamoxifen alone. Tamoxifen was given in a daily dose of 30 mg for one year.

All patients were followed according to a strict schedule including repeated clinical examinations, chest X-rays, bone scans and mammographic examinations of the remaining breast. Local and regional recurrences were verified by cytological or histopathological examinations. A diagnosis of distant recurrence was based on typical findings on X-ray examinations. Bone metastases were verified by radiological examinations guided by scintigraphic findings or by repeated bone scans showing progress. The site of recurrence was defined as the one first diagnosed. All patients with receptor positive tumors were given endocrine treatment upon relapse, either as sole therapy or in combination with other treatment modalities (surgery, radiotherapy or chemotherapy).

The trial started in January 1978 and was closed in November 1983 for premenopausal patients and in December 1984 for postmenopausal patients. The present data were evaluated in March 1985 at which time the median observation time was 39 (range 14-85 months) for premenopausal and 32 (range 2-86 months) for postmenopausal patients. A delay of reporting of follow-up data excluded 16 (3%) of the 539 patients from the present analysis.

Hormone receptor analyses

Biopsy material was obtained from the primary tumors by a standardized technique. The samples (100-400 mg) were cut from the tumors so that they should represent both central and peripheral parts of the tumor. The sample material was transported in solid CO₂ or fresh on ice to the receptor laboratory and kept in liquid nitrogen for not more than two weeks before analysis. Samples reaching the laboratory at an improper temperature, samples containing too small amounts of tissue (less than 0.5 mg protein/ml DCC treated supernatant) and samples from patients on hormonal treatment were excluded from the present study. All analyses were performed in the same laboratory.

The receptor determinations were performed as previously described (21). ER was assayed with isoelectric focusing originally elaborated by Wrangé et al. (22). PgR was determined with the multiple point dextran-coated charcoal (DCC) technique and Scatchard analysis. During the time of study, methodological improvements resulted in an increased sensitivity of receptor measurements and an increased recovery of ER and PgR. To include receptor values obtained before and after the methodological modifications, control experiments were performed using the two variants of methods in each sample. These experiments as well as the validity of

the conversion is described in more detail elsewhere (23). Cut-off points of 10 fmol specifically bound ^3H -estradiol/mg protein (ER) and 30 fmol specifically bound ^3H -R 5020/mg protein (PgR) were chosen to define ER and PgR positivity (ER+ and PgR+, respectively). Differences in recurrence free survival were also tested with varying cut-off points ranging from 0 to 100 fmol/mg protein.

The protein concentration was measured in the supernatant according to Lowry et al. (24).

Statistical analysis

For comparison of differences in RFS and survival, life table analysis with the generalized Wilcoxon test was applied. Comparisons of characteristics between different subgroups of patients were performed with the chi-squared test for contingency tables.

RESULTS

The 539 patients whose ER and PgR levels were determined were compared with the additional 584 patients without both ER and PgR data but included in the same trial of adjuvant therapy (Table 1). This comparison revealed no differences regarding age, lymph-node involvement or distribution between treatment groups. Tumors greater than 2 cm in diameter were, however, more often analyzed for receptors than tumors ≤ 2 cm.

Receptor distributions.

The ER and PgR status in samples among pre- and postmenopausal patients are given in Table 2. Table 3 gives the corresponding distribution of the combination of ER and PgR. The proportions of ER+ and PgR+ tumors were similar whether or not axillary nodes were involved (Table 4).

Table 1.
Characteristics of patients with and without receptor assay.

		Analyses of ER and PgR		No analyses	
		n	%	n	%
Premenop. arm	1	64	44.8	79	55.2
	2	61	41.5	86	58.5
	3	59	42.4	80	57.6
	1-3	184	42.9	245	57.1
Postmenop. arm	1	109	48.4	116	51.6
	2	114	49.1	118	50.9
	3	132	55.7	105	44.3
	1-3	355	51.2	339	48.8
Nodal status	N0	215	50.1	214	49.9
	N1-3	211	45.9	249	54.1
	N4+	113	48.3	121	51.7
Tumor size	≤ 2	177	37.0	302	63.0
	> 2	362	53.8	311	46.2
Mean age (years)	Pre	46.5		46.9	
	Post	62.5		62.6	

No significant differences regarding the distribution of ER status were found between the different treatment groups in pre- and postmenopausal patients. The same was true for the distribution of PgR status in premenopausal patients. However, among postmenopausal patients, 55% in the combined treatment group (radiotherapy + tamoxifen) were PgR+ compared to 30% in patients receiving only radiotherapy and to 36% in patients receiving tamoxifen only.

Premenopausal patients.

RFS. No significant differences between the receptor positive and negative groups were found with the chosen cut-off points of 10 (ER) and 30 (PgR) fmol/mg protein, respectively (Fig. 1 and 2). Nor was any difference in RFS noted when ER+/PgR+ patients were compared with ER-/PgR- patients. This pattern was similar in each subgroup according to the modality of postoperative treatment. However, by varying the cut-off points for both ER and PgR, significant differences in RFS could be observed (Table 5). The distribution of samples with ER

and PgR concentrations above the cut-off point, which demonstrated the strongest significance (50 fmol/mg protein) was 23% and 45%, respectively.

Survival. Statistically significant differences were observed in favour of receptor positive patients. ER+ patients had a longer survival than ER- patients (p=0.009) and the same was true when PgR+ patients were compared with PgR- patients (p=0.007). When ER+/PgR+ patients were compared with ER-/PgR- patients a highly significant difference in survival was also obtained (p=0.003). In premenopausal patients, survival after relapse was significantly longer in ER+ patients than in ER- patients (p=0.006).

Table 2.
Receptor status of pre- and postmenopausal patients.

	ER ¹				PgR ¹			
	<10		≥10		<30		≥30	
	n	%	n	%	n	%	n	%
Pre	80	43	104	57	92	50	92	50
Post	112	32	243	68	211	59	144	41

¹fmol/mg protein

Table 3.
Distribution of the combined ER and PgR status of pre- and postmenopausal patients.

	Premenopausal		Postmenopausal	
	n	%	n	%
ER-PgR-	65	35	107	30
ER+PgR-	27	15	104	29
ER-PgR+	15	8	5	1
ER+PgR+	77	42	139	39
	184		355	

Table 4.
Distribution of receptor status according to node involvement.

		ER ¹				PgR ¹			
		<10		≥10		<30		≥30	
		n	%	n	%	n	%	n	%
Pre	N0	28	45	34	55	33	53	29	47
	N+	52	43	70	57	59	48	63	52
Post	N0	48	31	105	69	90	59	63	41
	N+	64	32	138	68	121	60	81	40

¹fmol/mg protein

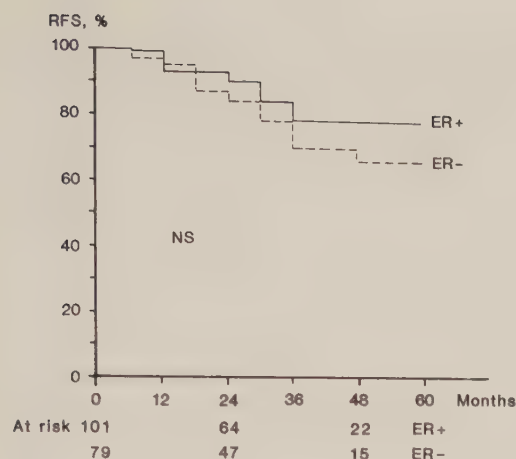


Figure 1.
Recurrence free survival (RFS) in premenopausal patients according to ER status.

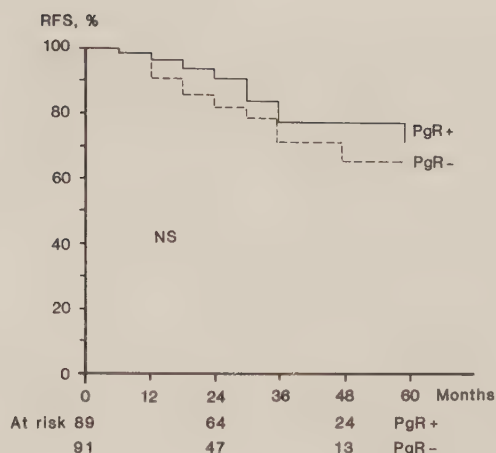


Figure 2.
Recurrence free survival (RFS) in premenopausal patients according to PgR status.

Table 5.
The influence of cut-off points on ER (a) and PgR (b) as prognostic indicators in premenopausal breast cancer patients (stage II). The differences in recurrence free survival between patients with tumors below and above the cut-off points are shown as p-values.

a/		b/	
Cut-off point	p-value	Cut-off point	p-value
ER ¹		PgR ¹	
0	0.38	0	0.49
5	0.28	10	0.31
10	0.23	20	0.42
20	0.25	30	0.067
30	0.15	40	0.034
40	0.027	50	0.0087
50	0.026	60	0.015
60	0.064	100	0.010
100	0.26		

¹ fmol/mg protein

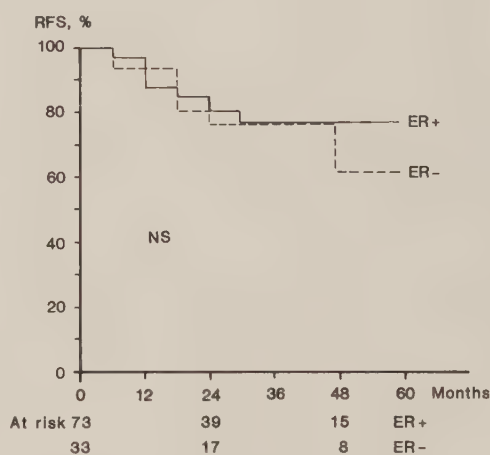


Figure 3.
Recurrence free survival (RFS) in postmenopausal patients not treated with adjuvant tamoxifen according to ER status.

Postmenopausal patients.

RFS. In postmenopausal patients not treated with adjuvant tamoxifen, no significant difference was observed between ER+ and ER- patients (Fig. 3). The same was true regarding both PgR and the combination of ER and PgR. Neither were any differences obtained when the cut-off points were varied between 0-100 fmol/mg protein as described above for premenopausal patients. In those postmenopausal patients who were treated with adjuvant tamoxifen (with or without postoperative radiotherapy) significant differences in RFS were observed between ER+ and ER- patients as well as between PgR+ and PgR- patients (Fig. 4 and 5). When ER+/PgR+ patients

were compared with ER-/PgR- patients the same marked difference persisted, but was not further pronounced. ER+/PgR+ patients also had a significantly better RFS than ER+/PgR- patients ($p=0.004$).

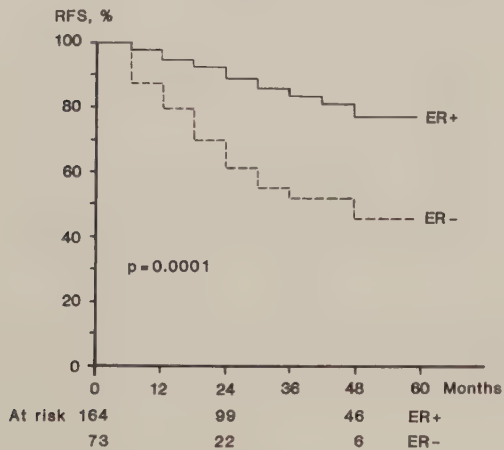


Figure 4.
Recurrence free survival (RFS) in tamoxifen treated postmenopausal patients according to ER status.

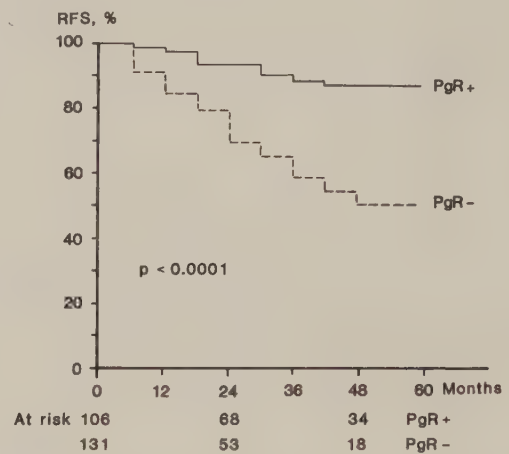


Figure 5.
Recurrence free survival (RFS) in tamoxifen treated postmenopausal patients according to PgR status.

Survival. In postmenopausal patients not treated with adjuvant tamoxifen, the survival was better in ER+ patients compared to ER- patients ($p=0.04$). This difference in survival was not observed between PgR+ and PgR- patients or when ER and PgR were combined. However, among postmenopausal patients treated with adjuvant tamoxifen significantly better survival was observed between ER+ and ER- patients as well as between PgR+ and PgR- patients (Fig. 6 and 7). The same was true when ER+/PgR+

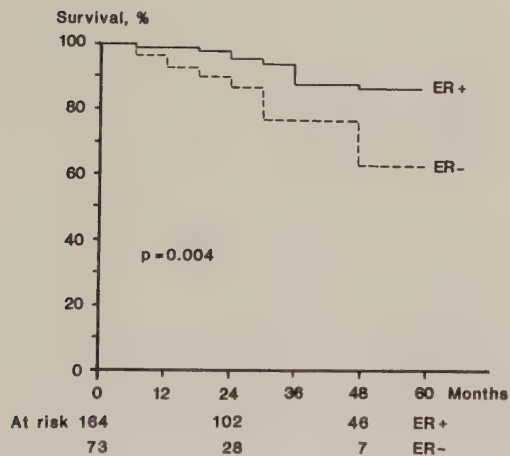


Figure 6.
Survival in tamoxifen treated postmenopausal patients according to ER status.

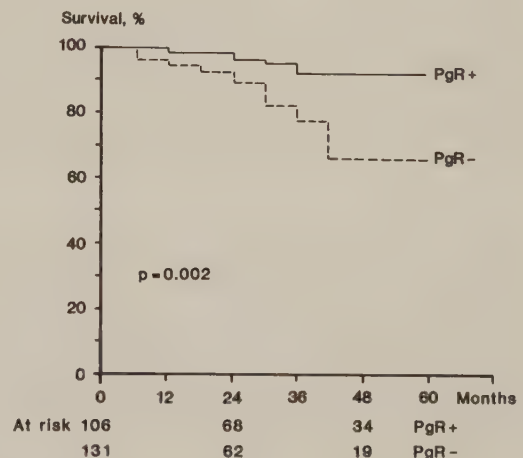


Figure 7.
Survival in tamoxifen treated postmenopausal patients according to PgR status.

patients were compared with ER-/PgR- patients. In postmenopausal patients, not treated with adjuvant tamoxifen, the number of deaths was too small to allow calculation of differences in survival after relapse. For patients treated with adjuvant tamoxifen no difference in survival after relapse according to receptor status was observed.

DISCUSSION

While there is a widespread agreement on the value of steroid receptors as predictors of response to endocrine treatment of patients with generalized disease, there is less certainty about the relations between these receptors and prognosis after treatment of primary breast cancer. A longer recurrence free survival (RFS) has been reported for patients with ER+ tumors (6-8). Two recent studies reported such a correlation only in node-positive patients (25) or in patients with four or more lymph nodes involved (26).

The present data do not suggest any correlation between RFS and ER or PgR status, alone or in combination, in premenopausal patients with the commonly applied cut-off points at our laboratory. The cut-off points (10 fmol ER/mg protein and 30 fmol PgR/mg protein) were chosen to get a receptor distribution similar to other investigators. Considering both ER and PgR together did not alter this observation. Our findings are in agreement with several other reports (10-14,27-28). However, by varying the cut-off points for ER and PgR, significant differences in RFS could be obtained in the present study. These differences were, however, found to be restricted to those premenopausal patients receiving adjuvant cyclophosphamide, alone or in combination with radiotherapy. This interesting observation can be attributed to endocrine effects of the chemotherapy, which is indicated by that the vast majority of patients treated with cyclophosphamide had amenorrhea during the time of treatment.

One explanation for the diverging results from different investigators concerning ER and PgR as independent prognostic indicators may thus be the choice of cut-off point. The importance of this has also been shown by Forrest et al. (29). Another possible explanation may be differences in methods for receptor assay. Different definitions regarding the date of relapse may also contribute to confusion. All patients in the present study were however followed according to the same strict protocol, which should minimize variations in this last respect.

Regarding survival in premenopausal patients, significant differences were observed in favour of receptor positive patients. This has also been

observed by others (7,18,27,30). This is probably explained by the higher frequency and the longer duration of response to endocrine treatment of metastatic disease in patients with receptor positive primary tumors, a hypothesis that is supported by the observation in the present study that survival after relapse was longer for receptor positive patients. Similar observations were made by Howell et al. (18).

In postmenopausal patients, not treated with adjuvant tamoxifen, no relationship was observed between receptor status and RFS. This finding persisted when the cut-off point was varied. The similarity between these patients and those premenopausal patients, not treated with adjuvant chemotherapy, is thus striking.

In those postmenopausal patients who were treated with adjuvant tamoxifen, highly significant differences in RFS were observed comparing receptor positive patients with receptor negative patients. These observations are in agreement with recent reports of two large trials on adjuvant tamoxifen treatment of postmenopausal patients (31,32). Another large trial on adjuvant tamoxifen treatment has, however, failed to show a relation between ER status and treatment response (17,33). In this trial, the assays were performed in several different laboratories, which may explain the diverging findings. The present study has also shown that additional prognostic information was obtained when also PgR was analyzed. This was demonstrated by the finding that a significant difference in RFS for postmenopausal patients treated with adjuvant tamoxifen was found between ER+/PgR+ patients and ER+/PgR- patients.

It should be stressed that our present data do not allow conclusions about the effects of adjuvant tamoxifen treatment in receptor negative patients, but strongly suggest that ER and PgR status are predictors of response to adjuvant tamoxifen treatment.

No difference in survival after relapse according to receptor status were observed among the patients treated with adjuvant tamoxifen. This probably reflects the decreased probability of response to endocrine treatment for metastatic disease in these patients. Definite conclusion must however await a longer follow-up.

In conclusion, the present study has for premenopausal patients demonstrated a relation between RFS and high receptor concentrations. For postmenopausal patients, however, this study does not support the hypothesis that hormone receptor values are independent predictors of prognosis in primary breast cancer, but may be valuable in predicting response to adjuvant endocrine treatment in postmenopausal patients with primary breast cancer.

ACKNOWLEDGEMENTS

The present investigation was in part supported by grants from the Swedish Cancer Society, the Swedish Medical Research Council, and from John and Augusta Persson's Foundation.

REFERENCES

1. Pearson OH, McGuire WL, Brodkey J, Marshall J. Estrogen receptors and prediction of the response of metastatic breast cancer to hypophysectomy. In: McGuire WL, Carbone PP, Vollmer EP (eds): Estrogen receptors in human breast cancer. Raven Press, New York 1975, 31-36.
2. Heuson JC, Longeval E, Mattheim WH, Deboel MC, Sylvester RJ, Leclercq G. Significance of quantitative assessment of estrogen receptors for endocrine therapy in advanced breast cancer. *Cancer* 1977, 39, 1971-1977.
3. Westerberg H, Nordenskjöld B, Wrange Ö, Gustafsson J-Å, Humla S, Theve N-O, Silverswärd C, Granberg P-O. Effects of antiestrogen therapy on human mammary carcinomas with different estrogen receptor content. *Eur J Cancer* 1978, 14, 619-622.
4. McGuire WL. An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. In: Jacobelli S et al. Hormones and Cancer. Raven Press, New York 1980, 337-343.
5. Bloom ND, Tobin EH, Schreiberman B, Degenshein GA. The role of progesterone receptors in the management of advanced breast cancer. *Cancer* 1980, 45, 2992-2997.
6. Knight WA, Livingston RB, Gregory EJ, McGuire WL. Estrogen receptor as an independent prognostic factor for early recurrence in breast cancer. *Cancer Res* 1977, 37, 4669-4671.
7. Hähnel R, Woodings T, Vivian AB. Prognostic value of estrogen receptors in primary breast cancer. *Cancer* 1979, 44, 671-675.
8. Godolphin W, Elwood JM, Spinelli JJ. Estrogen receptor quantitation and staging as complementary prognostic indicators in breast cancer: a study of 583 patients. *Int J Cancer* 1981, 28, 677-683.
9. Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS. Progesterone receptors as a prognostic factor in stage II breast cancer. *New Engl J Med* 1983, 309 (22), 1343-1347.
10. Hilf R, Feldstein ML, Gibson SL, Savlov ED. The relative importance of estrogen receptor analysis as a prognostic factor for recurrence or response to chemotherapy in woman with breast cancer. *Cancer* 1980, 45, 1993-2000.
11. Campbell FC, Blamey RW, Elston CW, Nicholson RI, Griffiths K, Haybittle JL. Oestrogen-receptor status and sites of metastasis in breast cancer. *Br J Cancer* 1981, 44, 456-459.
12. Shapiro CM, Schifeling D, Bitran JD, Desser RK, Rochman H, Michel A, Shapiro R, Evans R, Kozloff MF, Recant W, Billings AA. Prognostic value of the estrogen receptor level in pathologic stage I and II adenocarcinoma of the breast. *J Surg Oncol* 1982, 19, 119-121.

13. Howat JMT, Barnes DM, Harris M, Swindell R. The association of cytosol oestrogen and progesterone receptors with histological features of breast cancer and early recurrence of disease. *Br J Cancer* 1983, 47, 629-640.
14. Parl FF, Schmidt BP, Dupont WD, Wagner RK. Prognostic significance of estrogen receptor status in breast cancer in relation to tumor stage, axillary node metastasis, and histopathologic grading. *Cancer* 1984, 54, 2237-2242.
15. Fisher B, Redmond C, Brown A, Wickerham DL, Wolmark N, Allegra J, Escher G, Lippman M, Savlov E, Wittliff J, Fisher ER. Influence of tumor estrogen and progesterone receptor levels on the response to tamoxifen and chemotherapy in primary breast cancer. *J Clin Oncol* 1983, 1 (4), 227-241.
16. Ludwig Breast Cancer Study Group. Randomised trial of chemo-endocrine therapy, and mastectomy alone in postmenopausal patients with operable breast cancer and axillary node metastasis. *Lancet* 1984, i, 1256-1260.
17. Nolvadex Adjuvant Trial Organization. Baum M, Brinkley DM, Dossett JA, et al. Controlled trial on tamoxifen as adjuvant agent in management of early breast cancer. *Lancet* 1983, i, 257-261.
18. Howell A, Barnes DM, Harland RNL, Redford J, Bramwell VHC, Wilkinson MJS, Swindell R, Crowther D, Sellwood RA. Steroid hormone receptors and survival after first relapse in breast cancer. *Lancet* 1984, i, 588-591.
19. Aspegren K, Rydén S, Wiklander O. Experiences of a multicenter program on the treatment of mammary cancer stage II with a built in clinical trial of adjuvant chemotherapy or hormone therapy. *Acta Chir Scand suppl* 1979, 493, 125.
20. Arwidi A, Aspegren K, Augustsson N-E, Hafström Lo, Norgren A, Svahn-Tapper G. Postoperative radiation therapy in mammary carcinoma stage II. *Acta Radiol Oncol* 1979, 18, 273-281.
21. Norgren A, Borg A, Fernö M, Johansson U, Lindahl B, Tsiobanelis K. Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer. *Anticancer Res* 1982, 2, 315-320.
22. Wrangé Ö, Nordenskjöld B, Gustafsson J-A. Cytosol estradiol receptor in human mammary carcinoma: An assay based on isoelectric focusing in polyacrylamide gel. *Anal Biochem* 1978, 85, 461-475.
23. Fernö M. Thesis 1985, Infotryck, Lund, Sweden. In press.
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951, 193, 265-275.
25. Saez S, Cheix F, Mayer M. Estrogen and progesterone receptors as prognostic factors in early breast cancer. *Recent Results Can Res* 1984, 91, 192-198.
26. Kinne DW, Ashikari R, Butler A, Menendez-Botet C, Rosen PP, Schwartz M. Estrogen receptor protein in breast cancer as a predictor of recurrence. *Cancer* 1981, 47, 2364-2367.
27. Stewart JF, Rubens RD, Millis RR, King RJB, Hayward JL. Steroid receptors and prognosis in operable (Stage I and II) breast cancer. *Eur J Cancer Clin Oncol* 1983, 19 (10), 1381-1387.

28. Butler JA, Bretsky S, Menendez-Botet C, Kinne DW. Estrogen receptor protein of breast cancer as a predictor of recurrence. Cancer 1985, 55, 1178-1181.
29. Forrest APM, Black RB, Humeniuk V et al. Preoperative assessment and staging of breast cancer. J R Soc Med 1980, 73, 561-566.
30. Blamey RW, Bishop HM, Blake JRS et al. Relationship between primary breast tumor receptor status and patient survival. Cancer 1980, 46, 2765-2769.
31. Rose C, Thorpe SM, Andersen KW, Pedersen BV, Mouridsen HT, Blichert-Toft M, Rasmussen BB. Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer with high oestrogen receptor values. Lancet 1985, i, 16-19.
32. Stewart HJ, Prescott R. Adjuvant tamoxifen therapy and receptor levels. Lancet 1985, i, 573.
33. Baum M, Brinkley DM, Dossett JA et al. for the Nolvadex Adjuvant Trial Organisation. Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. Lancet 1985, i, 836-840.

APPENDIX

Participating institutions and principal investigators

Departments of Surgery

Halmstad	Lars Risholm
Helsingborg	Per-Ebbe Jönsson, Martin Malmberg
Hässleholm	Hans Danielsson
Karlshamn	Rutger Eriksson, Østen Nelsen
Karlskrona	Bengt Malmpor
Kristianstad	Ake Andersson, Mats Eriksson
Landskrona	Christian Kugelberg
Ljungby	Magnus Schwartz
Lund	Larsolof Hafström, Stefan Rydén
Malmö	Knut Aspegren, Ulf Ljungqvist
Simrishamn	Per Lilja, Ivar Spak
Trelleborg	Erik Olsson, Arne Weiber
Växjö	Lars Bergljung, Per Sassner
Ystad	Sven Lenninger
Ängelholm	Bengt-Ake Åkesson

Departments of Oncology

Lund	Torsten Andersson, Sven-Börje Ewers, Mårten Fernö, Dick Killander, Torgil Möller, Allan Norgren
Malmö	Stig Borgström, Torsten Landberg

Plasma steroid hormones, cytosol receptors, and thymidine incorporation rate in endometrial carcinoma

Bengt Lindahl, M.D., Per Alm, M.D., Mårten Fernö, B.A., Allan Norgren, M.D., and Claes Tropé, M.D.

Lund, Sweden

Histopathologically evaluated endometrial carcinomas were analyzed for the presence of estradiol and progesterone receptors and for tumor thymidine incorporation rate. Plasma estradiol and progesterone concentrations were also measured. With the use of an improved assay, all endometrial carcinomas were found to contain estradiol receptors; 81 of 92 (88%) contained measurable concentrations of progesterone receptors. The concentrations of both receptors were positively correlated to the degree of differentiation. The moderately and poorly differentiated tumors could be further divided into subgroups with low and high concentrations of receptors, which might be a reflection of an independent biologic characteristic of a tumor. These subgroups did not differ in any of the other variables studied, including the age of the patients. The plasma concentrations of estradiol showed a positive correlation to the degree of differentiation and to the concentration of both receptors. It is suggested that improved analyses of estradiol and progesterone receptors in endometrial carcinomas may add information to the conventional histopathologic and clinical classification which will be of significant value in the proper treatment of the disease. (AM. J. OBSTET. GYNECOL. 149:607, 1984.)

Steroid hormones and their receptors in normal or malignant endometrium have been thoroughly investigated. Estrogen induces uterine growth^{1, 2} and stimulates the synthesis of receptor proteins for both estradiol³ and progesterone.^{4, 5} Estrogens are also believed to promote endometrial cancer, since women exposed to estrogen exhibit a higher risk of developing endometrial cancer.^{6, 7} Progesterone opposes the effects of estrogen on receptor synthesis.⁴ Furthermore, in patients with advanced endometrial cancer, progesterone treatment results in objective remission, in which well-differentiated tumors respond better than poorly differentiated ones.⁸ In an earlier investigation⁹ significantly higher concentrations of both estradiol receptors and progesterone receptors were found in well-differentiated and moderately differentiated tumors than in poorly differentiated ones. However, there was a wide variation of receptor concentrations within the three groups of tumors, which might be one of the explanations of why some well-differentiated tumors respond poorly to hormonal therapy whereas some poorly differentiated tumors respond quite well.

In a previous report⁹ estradiol and progesterone re-

ceptors could only be measured on different samples from the same tumor. Therefore the concentrations of estradiol and progesterone receptors might not have been strictly comparable. Furthermore, sometimes measurement of progesterone receptors had to be abandoned since relatively large amounts of tumor materials were required.

Relatively small modifications of the assay techniques showed that both estradiol and progesterone receptors could be assayed in cytosol prepared from the same small (40 to 400 mg) sample of tumor.¹⁰ As an additional advantage, usually both receptors could be assayed. Correlation with plasma concentration of steroids and, when possible, with tumor thymidine incorporation rate was also made.

Material and methods

Curettage materials of 93 endometrial carcinomas were obtained from women referred for primary treatment to the Gynecological Section, Department of Oncology, University Hospital, Lund, Sweden, from March, 1980, to April, 1982.

All patients had previously been subjected to a first diagnostic curettage at other hospitals. A second curettage was performed 2 to 3 weeks later to collect material for analysis immediately prior to the first intra-uterine packing. The curettage material was minced, mixed, and immediately divided into different portions for further processing.

Parts of the materials were fixed in neutral formol for histopathologic evaluation,¹¹ which was always performed by the same pathologist.

From the Department of Obstetrics and Gynecology, the Department of Pathology, and the General and Gynecological Sections of the Department of Oncology, University Hospital.

This study was supported in part by grants from the Swedish Cancer Society and John and Augusta Persson's Foundation.

Received for publication June 21, 1983; revised October 25, 1983; accepted January 27, 1984.

Reprint requests: Bengt Lindahl, M.D., Department of Obstetrics and Gynecology, University Hospital, S-221 85 Lund, Sweden.

Table I. Percent of progesterone receptors—positive endometrial tumors according to degree of differentiation

Differentiation	No. of tumors analyzed	Progesterone receptors—positive tumors	
		n	%
Well differentiated	22	21	95
Moderately differentiated	45	44	98
Poorly differentiated	25	16	64

Preparation of cytosol and receptor measurements were made as described previously.¹⁰ Concentrations of estradiol receptors were assayed with isoelectric focusing and concentrations of progesterone receptors were assayed by a multiple-point dextran-coated charcoal technique; all concentrations were calculated as femtomoles of specifically bound ligand per milligram of tissue by wet weight, per milligram of cytosol protein, and per milligram of DNA of the homogenate.

In 29 patients the rate of tritium-labeled thymidine incorporation into endometrial cells was calculated according to the method described by Nordqvist.¹² The concentrations of estradiol and progesterone in plasma were determined according to the methods of Lindberg et al.¹³ and Youssefnejadian et al.¹⁴

Results

All of the 92 tumors analyzed were estradiol receptors—positive (100%). Corresponding figures for progesterone receptors were 81 positive of 92 analyzed tumors (88%). The positivity of progesterone receptors was significantly related to tumor differentiation ($p < 0.001$, Fisher's exact test). Nearly 100% of well-differentiated and moderately differentiated tumors had progesterone receptors, compared to 64% of the poorly differentiated tumors (Table I).

Tables II and III summarize the mean values of estradiol and progesterone receptors calculated as femtomoles per milligram of protein or DNA in relation to the degree of tumor differentiation. It was significantly found ($p < 0.001$, analysis of variance) that poorly differentiated tumors had the lowest values for both estradiol and progesterone receptors and well-differentiated tumors had the highest receptor values. When $^3\log$ values were used, poorly differentiated tumors were shown to have lower values than both moderately differentiated and well-differentiated tumors ($p < 0.001$); moderately differentiated tumors had lower values than well-differentiated ones ($p < 0.05$) but only when values for estradiol receptors were calculated as femtomoles per milligram of DNA.

The distribution of $^3\log$ values for concentration of estradiol receptors (estradiol receptor per milligram of DNA) is shown in Fig. 1. The 25 poorly differentiated tumors can be subjectively divided into two separate groups with respect to the concentration of estradiol receptors. One group consists of the 13 tumors with the lower values, whereas the other group is made up of the remaining 12 tumors with the higher concentration. Likewise, the $^3\log$ values for the concentration of progesterone receptors (shown in Fig. 2) reveal a pattern of distribution similar to that of the values for estradiol receptors. Further, all except one of the poorly differentiated tumors were found to be in the lower grouping of concentrations of both estradiol receptors and progesterone receptors.

Among the moderately differentiated tumors we could subjectively identify four tumors with concentrations of estradiol receptors as low as those in the group of 13 poorly differentiated tumors (Fig. 1). For the concentrations of progesterone receptors (Fig. 2) an analogue pattern was found, but among the six moderately differentiated tumors with low concentrations of progesterone receptors only three tumors also had low concentrations of estradiol receptors. These small groups of tumors were responsible for the statistical difference ($p < 0.05$) between well-differentiated and moderately differentiated tumors when, as indicated in Figs. 1 and 2, receptor concentration was related to DNA only.

In the poorly differentiated and moderately differentiated tumors the mean values of the two groups with high and low receptor values are statistically different only with respect to values of estradiol and progesterone receptors but not with respect to other variables studied (plasma estradiol and progesterone concentrations, thymidine incorporation rate, and age).

The plasma concentrations of estradiol and progesterone are shown in Table IV. With the use of Spearman's rank correlation test there was a positive correlation between plasma estradiol concentration and degree of tumor differentiation ($p < 0.05$). Further, the plasma estradiol concentration also showed positive correlation with estradiol receptors (measured as femtomoles per milligram of protein or milligram of wet weight; $p < 0.05$) and an even better correlation with progesterone receptors ($p < 0.001$). No correlation was found between plasma estradiol and plasma progesterone concentrations. Between plasma estradiol concentration and thymidine incorporation rate there was a weak negative correlation ($p < 0.04$). Plasma progesterone concentrations did not correlate with any other variable studied.

The thymidine incorporation rate is shown in Table V. Although the rate is increasingly higher in poorly differentiated tumors, the standard deviation is so high

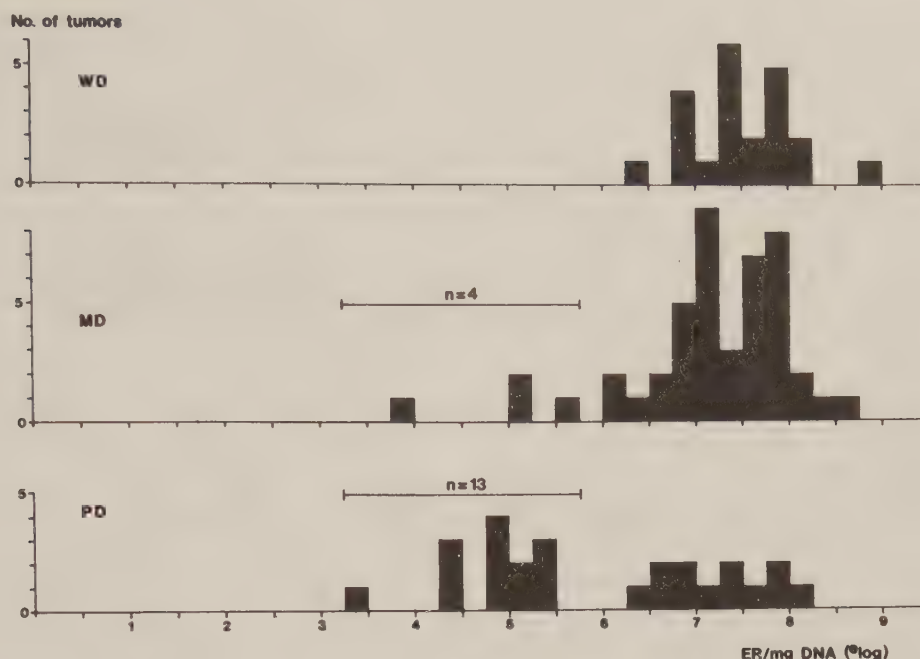


Fig. 1. Histogram showing distribution of estradiol receptors (ER) concentration ($\log$) in 92 well-differentiated (WD), moderately differentiated (MD), and poorly differentiated (PD) endometrial carcinomas. In the moderately differentiated and poorly differentiated groups there were four and 13 tumors, respectively. These 17 tumors were responsible for the main differences in the mean concentration of estradiol receptors among the three groups of tumors. For details, see Results.

Table II. Concentrations of estradiol receptors and progesterone receptors according to differentiation of endometrial tumors

Differentiation	n	Estradiol receptors (fmol/mg protein)		Progesterone receptors (fmol/mg protein)	
		Mean	SD	Mean	SD
Well differentiated	22	281	387	505	423
Moderately differentiated	45	160	118	452	485
Poorly differentiated	25	56	47	93	137

Table III. Concentrations of estradiol receptors and progesterone receptors according to differentiation of endometrial tumors

Differentiation	n	Estradiol receptors (fmol/mg DNA)		Progesterone receptors (fmol/mg DNA)	
		Mean	SD	Mean	SD
Well differentiated	22	2138	1530	5677	4432
Moderately differentiated	45	1682	1075	4232	4097
Poorly differentiated	24	829	897	1301	1910

that no statistical significance was found with this small number of patients. In the study material the thymidine incorporation rate and progesterone receptors showed a weak negative correlation ($p < 0.04$).

The age distribution of patients within this study is shown in Table VI. No statistical differences were

found between the age of patients and tumor differentiation.

Comment

As in the case of breast cancer,¹⁰ some adaptations of routine receptor assays made it possible to measure

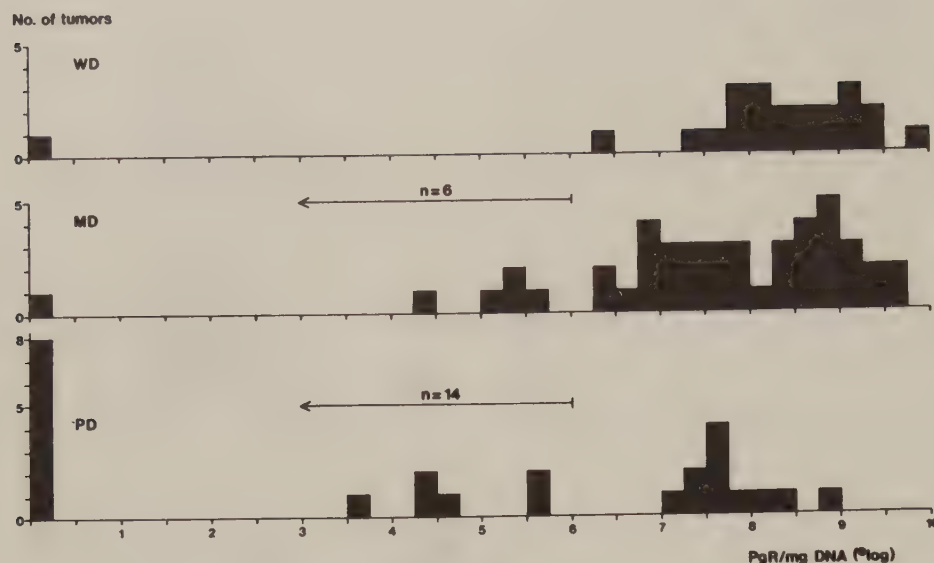


Fig. 2. Histogram showing distribution of progesterone receptors (PgR) concentration ($^{\circ}\log$) in the same 92 tumors shown in Fig. 1. The 20 tumors in the moderately differentiated and poorly differentiated groups were responsible for the main differences of mean concentration of progesterone receptors among the three groups. For details, see Results.

Table IV. Plasma estradiol and progesterone concentrations according to differentiations of endometrial tumors

Differentiation	n	Estradiol (pg/ml)		Progesterone (ng/ml)	
		Mean	SD	Mean	SD
Well differentiated	18	57.6	42	0.193	0.52
Moderately differentiated	40	50.1	34	0.165	0.45
Poorly differentiated	22	31.5	15	0.072	0.22

both estradiol and progesterone receptors in the same endometrial tumor cytosol preparation. Obvious advantages were achieved from a practical point of view and with regard to receptor recovery. With use of the present procedure the limit of detection of estradiol receptors was in the order of 1.0 fmol/ml of cytosol, and the limit of detection of progesterone receptors was 5 to 10 fmol/ml. The assay procedure developed was applied to 92 endometrial carcinomas, and adequate quantities of cytosol were recovered to permit the assay of both estradiol and progesterone receptors. In comparison, it may be mentioned that in the previous study in which estradiol and progesterone receptors were assayed in different samples of the same tumor,⁹ assay of progesterone receptors was applied to 88% of the tumors. Estradiol receptors were found in all 92 tumors in this study compared to a finding of 97 of 106 (92%) in the previous work. On the other hand, the percentage of progesterone receptors-positive tumors was approximately the same (88%), but it should be recalled that analysis for progesterone receptors was

now made in all of the samples. In the two different series of assays the receptor concentrations were also compared and were found to be increased two to three times. It seems evident that the improved procedure developed for breast cancer was advantageous for endometrial carcinomas also and resulted in increased receptor recovery.

The percent of progesterone receptors-positive tumors was higher in the well-differentiated and moderately-differentiated tumor groups than in the poorly differentiated group and was not related to age. The trend toward fewer numbers of progesterone receptors-positive tumors in the poorly differentiated group is similar to the results of Ehrlich et al.,¹⁵ but the number of positive tumors is higher than earlier reported. The preponderance of progesterone receptors-negative tumors in the poorly differentiated group may in part be a reflection of the special properties of the receptor-ligand complex and of insufficient techniques for the detection of progesterone receptors.

The present data show that the concentrations of

estradiol and progesterone receptors are higher in the more developed tumors. With the used %log data, the moderately differentiated and poorly differentiated tumors could be subdivided into two groups, with high and low concentrations of receptors, respectively. It should be noted also that no well-differentiated tumor was found in the low-concentration group, but 13 of the 25 poorly differentiated tumors were of low concentration. Among the 45 moderately differentiated tumors, four showed a low concentration of estradiol receptors and six a low concentration of progesterone receptors. The low- and high-concentration groups did not differ in any other variable investigated in this study (plasma estradiol and progesterone concentrations, thymidine incorporation rate, and age).

It has been observed that (1) receptor concentration correlated to tumor differentiation, (2) moderately differentiated and poorly differentiated tumors could be divided with respect to receptor concentration into two subgroups, and (3) these subgroups did not differ in any other variable studied. These observations indicate that receptor concentration is an expression of an important, independent biologic characteristic of a tumor.

In a study of 777 patients with endometrial carcinoma the 5-year death rate and occurrence of distal metastases were highest in patients with poorly differentiated tumors as compared to those with moderately differentiated and well-differentiated tumors.¹⁶ In the present investigation three groups of differentiation were used, and a subgroup of the moderately differentiated tumors was identified according to receptor status. This subgroup had lower receptor concentrations than the well-differentiated tumors. Whether this group of moderately differentiated tumors represents patients with a more unfavorable prognosis will be a matter of special interest in the long-term follow-up program for the patients in this study.

In the present study plasma concentration of estradiol correlated well to tumor differentiation ($p < 0.01$). The plasma estradiol also showed a positive correlation with concentrations of estradiol and progesterone receptors in the tumors. These findings are not in accord with those of other workers.¹⁷

The heterogeneous nature of most tumors is well known.¹⁸ The heterogeneity might also involve steroid receptor synthesis. Thus absence of receptors in one part of a tumor selected for assay does not exclude the presence of receptors within another part, which then might be subject to study in other respects. For this reason it seems to be of great importance to use minced tumor material to obtain the average tumor values for the variables as was done in the present study. This information may have a special practical importance for the measurements of progesterone receptors because of the inherent difficulties mentioned above. The

Table V. Thymidine incorporation rate according to differentiation of endometrial tumors

Differentiation	n	Thymidine incorporation* (%)	
		Mean	SD
Well differentiated	5	30	23.5
Moderately differentiated	18	44	41.1
Poorly differentiated	6	56	35.4

*No statistical significance.

Table VI. Age distribution of patients according to differentiation of endometrial tumors

Differentiation	n	Age* (yr)	
		Mean	SD
Well differentiated	22	61	9.5
Moderately differentiated	46	67	12.3
Poorly differentiated	25	65	9.4

*The age differences are not statistically different.

weak negative correlation between thymidine incorporation rate and concentration of progesterone receptors in contrast to the positive correlation noticed previously might be explained by the difference in handling of the tumor material as well as the limited number of samples available for thymidine incorporation studies.

We are most grateful to Bo Gullberg, statistical advisor of the South Swedish Regional Tumour Registry, University of Lund, Sweden, for advice on the statistical treatment of the data.

REFERENCES

1. Jensen EV, Jacobson HI. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res 1962;18:387.
2. Andersson JN, Peck EJ Jr, Clark JH. Estrogen-induced uterine responses and growth: relationship to receptor estrogen binding by uterine nuclei. Endocrinology 1975; 96:160.
3. Hoffman PG, Siiteri K. Sex steroid receptors in gynecologic cancer. Obstet Gynecol 1980;55:648.
4. Thi MTL, Baulieu EE, Milgrom E. Comparison of the characteristics and of the hormonal control of endometrial and myometrial progesterone receptors. J Endocrinol 1975;66:349.
5. Pollow K, Schmidt-Gollwitzer M, Nevinny-Stickel J. Progesterone receptors in normal human endometrium and endometrial carcinoma. In: McGuire WL, Raymond P, Baulieu G, eds. Progesterone receptors in normal and neoplastic tissues. New York: Raven Press, 1977.
6. Hulka BS, Fowler WC Jr, Kaufman DG, Grimson RC, Greenberg BG, Hogue CJR, Berger GS, Pulliam CC. Estrogen and endometrial cancer: cases and two control groups from North Carolina. AM J OBSTET GYNECOL 1980;137:92.
7. Jelovsek FR, Hammond CB, Woodard BH, Draffin R, Lee KL, Creasman WT, Parker RT. Risk of exogenous estrogen therapy and endometrial cancer. AM J OBSTET GYNECOL 1980;137:85.
8. Malkasian GD Jr, Decker DG, Mussey E, Johnsson CE.

- Progestogen treatment of recurrent endometrial carcinoma. *AM J OBSTET GYNECOL* 1971;110:15.
9. Lindahl B, Alm P, Borg Å, Fernö M, Grundsell H, Johnsson J-E, Norgren A, Tropé C. Correlation between estradiol 17 β and progesterone cytosol receptor concentration, histologic differentiation and ³H-thymidine incorporation in endometrial carcinoma. *Anticancer Res* 1982;2:203.
 10. Norgren A, Borg Å, Fernö M, Johansson U, Lindahl B, Isibanelis K. Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer. *Anticancer Res* 1982;2:315.
 11. Hendrickson MR, Kempson RL. Surgical pathology of the uterine corpus. In: Bennington JL, ed. *Major problems in pathology*. vol. 12, Philadelphia: WB Saunders, 1980.
 12. Nordqvist S. Hormonal responsiveness of human endometrial carcinoma studied in vitro and in vivo [Thesis]. Lund, Sweden: University of Lund, 1969.
 13. Lindberg BS, Lindberg P, Martinsson K, Johansson EDB. Radioimmunological methods for the estimation of oestrone, oestradiol-17 β and oestriol in pregnancy plasma. *Acta Obstet Gynecol Scand* 1974;32(suppl):5.
 14. Youssefnejadian E, Florensa E, Collins WP, Sommerville JF. Radioimmunoassay of plasma progesterone. *J Steroid Biochem* 1972;3:893.
 15. Ehrlich CE, Young PCM, Cleary RE. Cytoplasmic progesterone and estradiol receptors in normal, hyperplastic, and carcinomatous endometria: therapeutic implications. *AM J OBSTET GYNECOL* 1981;141:539.
 16. Grundsell H, Henriksson H, Johnsson J-E, Laurin J, Tropé C. Histological grading and treatment failures in carcinoma of the uterine body. *Ann Chir Gynaecol* 1980;69:65.
 17. Jänne O, Kauppila A, Kontula K, Syrjälä P, Vihko R. Female sex steroid receptors in normal, hyperplastic and carcinomatous endometrium: the relationship to serum steroid hormones and gonadotropins and changes during medroxyprogesterone acetate administration. *Int J Cancer* 1979;24:545.
 18. Aspegren K, Biörklund A, Tropé C. Heterogeneity in in vitro response to progesterone and melphalan of mammary tumours induced in the rat by 7,12-DMBA. *Acta Pathol Microbiol Scand [A]* 1976;84:297.

Antiestrogen Binding Sites in Human Breast Cancer Biopsies.

Measurement, Ligand-Specificity and – Affinity, and Correlation to Estrogen and Progesterone Receptors

MÅRTEN FERNÖ and ÅKE BORG

Department of Oncology, University Hospital, S-221 85 Lund, Sweden

Abstract. Specific binding sites for the antiestrogen tamoxifen ($AEBS_{tot}$), as measured with the dextran-coated charcoal technique and Scatchard analysis, were quantitated in 43 of 44 breast cancer samples (41-2,100 fmol/mg protein). In this work, $AEBS_{tot}$ has been defined as the sum of $AEBS_{spec}$ and estrogen receptor (ER). $AEBS_{spec}$ shows a very weak positive correlation with ER, and no correlation with progesterone receptor. Tamoxifen (TAM) has a lower affinity to ER compared with one of its main metabolites, 4-OH-TAM. This may be one reason why inhibition by excess estradiol on 3H -4-OH-TAM binding was better correlated with ER content in the same sample than if 3H -TAM was the radioactive ligand. It is therefore suggested that 3H -4-OH-TAM is a better choice than 3H -TAM for measuring $AEBS_{spec}$ alone. A possible role of $AEBS_{spec}$ for the clinical effects of TAM is discussed.

The antiestrogen tamoxifen (TAM) is widely used in the treatment of metastatic breast cancer. Response rate is positively correlated with the presence of estrogen receptor (ER) in the cytoplasm (1). Most of the anti-tumoural properties of TAM have also been explained by an interference with ER (2). About 50% of ER positive tumours do not, however, respond to TAM, whereas approximately 10% of ER negatives do so (1). No generally accepted explanation of this disagreement has as yet been presented, although several possible reasons have been given (3).

By using 3H -TAM *in vitro*, a further binding site for TAM, other than that of ER, the antiestrogen binding site ($AEBS_{spec}$) has been demonstrated (4). $AEBS_{spec}$ binds triphenylethylene antiestrogens but, in contrast to ER, it neither binds steroidal nor non-steroidal estrogens (5,6). $AEBS_{spec}$ has been found in ER positive human mammary carcinoma biopsies (7), and in ER negative as well as ER positive breast cancer cell-lines (8). It is not known whether and to what extent the presence of $AEBS_{spec}$ could explain the discrepan-

cy between tumour ER content and the response to TAM treatment. In the present investigation, the binding of 3H -TAM and 3H -4-OH-TAM (one of its main metabolites) has been measured in 20,000xg supernatants from human breast cancer biopsy homogenates and compared with the contents of ER and progesterone receptor (PgR) in the same samples.

Earlier studies have been mostly concerned with breast cancer cell-lines (8, 9, 10, 11) or different animal tissues (5, 6, 9, 12). Breast cancer biopsies have been examined in some studies, but only in a smaller number of samples (5, 7). The correlation with PgR has not been performed previously, which is also the case for the influence of an excess of estradiol on 3H -TAM or 3H -4-OH-TAM binding in relation to ER content in the same sample. Also, the binding of 3H -4-OH-TAM has hitherto been given only minor interest (10, 13). The relative binding affinities of TAM, its main metabolites (4-OH-TAM and N-d-Me-TAM) and estradiol to $AEBS_{spec}$ and ER, respectively, have also been studied.

Materials and Methods

Tissue. Biopsy material from human breast cancer was obtained at operation. It was then freed from fat, blood clots, etc., minced and kept at either $-70^{\circ}C$ or in liquid nitrogen until analysis. The binding of 3H -antiestrogens (3H -TAM and 3H -4-OH-TAM) was, in addition to the routine measurement of ER and PgR (14), determined only in those samples where adequate tissue material was obtained.

Chemicals. All chemicals were of analytical grade.

Ligands. 3H -Promegestone (3H -R 5020, 81-87 Ci/mmol), non-radioactive promegestone and 3H -trans-tamoxifen (3H -TAM, 71 Ci/mmol) were purchased from New England Nuclear (Boston, Ma, USA). 3H -estradiol (91-115 Ci/mmol), Z-4-hydroxy(N-methyl- 3H)-tamoxifen (3H -4-OH-TAM, 76 Ci/mmol) and non-radioactive Z-4-hydroxy-tamoxifen (4-OH-TAM), were obtained from Amersham International (Buckinghamshire, England). Non-radioactive trans-TAM (TAM) and N-desmethyl-tamoxifen (N-d-Me-TAM) were generously supplied by Imperial Chemical Industries (Macclesfield, Cheshire, England). Progesterone, 17 β -estradiol, estrone, dexamethasone and diethylstilbestrol were obtained from Sigma Chemical Company (St Louis, Mo, USA), and 5 α -dihydrotestosterone was delivered by Merck (Darmstadt, FRG).

Buffer solutions, preparation of 20,000xg supernatant, ER and PgR

Key Words: Antiestrogen binding sites, breast cancer, estrogen and progesterone receptors.

determinations, and protein measurement were performed exactly as previously described (14). ER was measured with isoelectric focusing (IF, unless otherwise indicated), whereas PgR was analyzed with the multiple point dextran-coated charcoal method (DCC) and Scatchard analysis. The level of sensitivity for ER and PgR was 1.0 fmol/ml and 10 fmol/ml, respectively (14). Protein measurement was performed according to Lowry *et al* (15).

The binding of ^3H -TAM and ^3H -4-OH-TAM was determined with the DCC technique and Scatchard analysis according to the following brief description. Aliquots of supernatant (after 20,000xg) were mixed with eight different concentrations, 0.06-8.0 nM (final concentrations), of ^3H -ligand dissolved in absolute ethanol (final ethanol concentrations=3%). For the determination of unspecific binding, samples were incubated with the strongest radioactive ligand concentration plus a hundred times excess of unlabelled ligand. In addition, the inhibition of a 100-fold excess of estradiol on the strongest radioactive concentration was studied for comparison with ER content as measured with IF in the same sample. After two hours incubation at 0°C, excess of ligand was absorbed with DCC (dextran: charcoal 0.08:0.80 (mg/ml), final concentrations). Thirty minutes of DCC treatment at 0°C was followed by centrifugation at 1,000xg, whereupon the counting of radioactivity and the calculation of specifically bound ^3H -ligand concentrations and K_d -values were performed as previously described (14).

The mean protein concentration in the incubation tubes was 2.9 ± 0.9 mg/ml. The addition of dimethylformamide to the buffer, used by some investigators to increase the solubility of these antiestrogenic ligands (8, 12), has not been found to be of any significance in our systems.

Relative binding affinity. Competition studies were performed to study the ability of estradiol, TAM, 4-OH-TAM, and N-d-Me-TAM to compete for the binding sites of ^3H -estradiol, ^3H -TAM and ^3H -4-OH-TAM, respectively. Supernatants after 20,000xg centrifugations were incubated with ^3H -estradiol (5.0nM), ^3H -TAM (8.0nM) or ^3H -4-OH-TAM (8.0nM), together with increasing concentrations (0-50 μM , 0-8.0 μM or 0-8.0 μM , respectively) of non-radioactive ligands for two hours at 0°C. Specifically bound ^3H -ligand was then separated from non-specifically bound by a DCC treatment (see above). Data were plotted as the per cent of total bound radioactive ligand versus the $^{10}\log$ -concentration of unlabelled competitive ligand. The relative binding affinities (RBA) were calculated from the amount of non-radioactive ligand required to displace 50 per cent of the specifically bound radioactive tracer.

Statistics. Correlations were calculated according to onesided Spearman's rank-correlation and p-values above 0.05 were considered statistically non-significant.

Results

AEBS_{tot} concentration, K_d -value, correlation with ER and PgR. In 43 out of 44 breast cancer samples from the 20,000xg supernatants, AEBS_{tot} (AEBS_{tot}=total number of antiestrogen binding sites, as measured with ^3H -TAM) was demonstrated. AEBS_{tot} consists of ER and another binding site (AEBS_{spec}) for the antiestrogen TAM and its main metabolites (4-OH-TAM and N-d-Me-TAM). AEBS_{tot} concentrations ranged between 41-2,100 fmol specifically bound ^3H -TAM/mg protein (Figure 1). The mean concentration ($\pm$ standard deviation (S.D.)) and median value were 1000 ± 600 and 990, respectively. The dissociation constant (K_d) ranged from 0.26 to 7.7 nM; ($2.1 \pm 1.8\text{nM}$), indicating that AEBS_{spec}, as well as steroid receptors, is a high-affinity binding site. No correlation between the K_d -values and the

concentration values was found ($r_s=0.24$; $p=0.063$). The mean concentrations ($\pm$ S.D.) of ER and PgR in these 44 samples were 100 ± 180 and 240 ± 580 fmol/mg protein, respectively. Eight of nine ER negative and 18 of 19 PgR negative samples contained AEBS_{tot} and for all of the 44 samples, only a very weak positive correlation with ER ($r_s=0.26$; $p=0.041$) was found, and no correlation between AEBS_{tot} and PgR ($r_s=0.12$; $p=0.24$). When only ER or PgR positive samples were examined, the coefficients were 0.33 ($p=0.026$; $n=35$) and -0.023 ($p=0.46$; $n=20$), respectively.

Effects of excessive estradiol on ^3H -TAM binding. Since ER is one of the binding sites for TAM, it was of interest to study the influence of a 100-fold excess of estradiol on ^3H -TAM binding. If this influence shows a good correlation with ER content in the same sample, it should be possible, by subtracting the amount of ^3H -TAM which binds to ER from AEBS_{tot}, to get the concentration of AEBS_{spec} alone. This comparison has been performed when all of the samples were included, but also when the samples were divided into two groups: group 1/ samples ($n=6$) with a relatively high ER content compared with AEBS_{tot} (e.g. ER/AEBS_{tot} ≥ 0.20); group 2/ samples ($n=37$) with a comparatively lower ER content (e.g. ER/AEBS_{tot} < 0.20). The correlation coefficient between the inhibition of excessive estradiol on ^3H -TAM binding and ER content was 0.31 ($p=0.023$) when all of the samples were included. This agreement was, however, due to the six samples from group 1 ($r_s=0.66$; $p=0.078$). The remaining 37 samples (group 2) showed no correlation ($r_s= -0.16$; $p=0.17$). Among these 37 samples, five of 28 ER positives (32-130 fmol ER/mg protein as measured with IF) showed no inhibition on ^3H -TAM binding by an excess of estradiol. On the contrary, seven of nine samples with very low or non-detectable ER levels, as measured with IF, showed an inhibition of at least 10 per cent (calculated to 39-280 fmol/mg protein). These experiments demonstrated that the inhibition of estradiol on ^3H -TAM binding only correlated with ER in the few (6/43) samples with a relatively high ER content, compared with AEBS_{tot}. Consequently, this does not seem to be a suitable method for measuring the concentration of AEBS_{spec} alone.

The relative binding affinity (RBA) of estradiol and antiestrogens to AEBS_{spec}. The RBA (for explanation, see Materials and Methods) of estradiol, TAM, 4-OH-TAM and N-d-Me-TAM to AEBS_{spec}, as measured with ^3H -TAM, was studied in three ER negative breast cancer biopsies. ER negative samples were chosen in order to avoid interference from TAM binding to ER. Figure 2 shows that estradiol, even at a 1000-fold excess, did not inhibit the specific binding of ^3H -TAM (nor did other steroids or non-steroidal estrogens, e.g. dexamethasone, progesterone, 5α -dihydrotestosterone, estrone, or diethylstilbestrol (data not shown)). Figure 2 also demonstrates that TAM is a more effective inhibitor of ^3H -TAM binding than 4-OH-TAM and N-d-Me-TAM. When calculating RBA from these three experiments as the mean

value, the RBA to $\text{AEBS}_{\text{spec}}$ for TAM, 4-OH-TAM and N-d-Me-TAM was 100%, 16% (11-23%) and 9.3% (6.3-12%), respectively. At about a 100-fold excess, all the antiestrogen ligands showed almost the same inhibition of ^3H -TAM binding. In order to investigate whether a longer incubation time (20 hours) influenced these results, a comparison between two and 20 hours was performed. One significant difference seen when using the longer incubation time was that the RBA of 4-OH-TAM increased to the same order as that of TAM (2 experiments, data not shown).

RBA of estradiol and antiestrogens to ER. The affinities of estradiol, TAM, 4-OH-TAM, and N-d-Me-TAM to ER were studied in five ER positive (>90 fmol ER/mg protein) breast cancer samples by their capacity to inhibit ^3H -estradiol binding (Figure 3). When calculating RBA from these five experiments, it was found that, when compared with estradiol (100%), TAM and N-d-Me-TAM have a much lower RBA, 0.84% (0.60-1.7%) and 0.69% (0.40-1.4%), respectively, to ER than has 4-OH-TAM (47%, 31-63%). At a 10,000-fold excess, all of the ligands completely inhibited the specific ^3H -estradiol binding. As these five experiments were performed with 20,000xg supernatants, where also $\text{AEBS}_{\text{spec}}$ is present, it might be possible that the RBA-values of the antiestrogens to ER could be influenced by their binding to $\text{AEBS}_{\text{spec}}$. When comparing with cytosols from 100,000xg centrifugations, however, where $\text{AEBS}_{\text{spec}}$ mainly is pelleted, no differences in RBA-values were found (4 experiments, data not shown). In control experiments it was also shown that no differences were obtained when comparing the longer incubation time (20 hours) with the shorter (2 hours) used in this study (2 experiments, data not shown).

A comparison between the specific binding of ^3H -TAM and ^3H -4-OH-TAM. As TAM and 4-OH-TAM bind both to $\text{AEBS}_{\text{spec}}$ and to ER, although with different affinities, it was of interest to compare the direct binding of these two radioactive ligands. A very high correlation ($r_s=0.93$; $p<0.001$) was found between ^3H -TAM and ^3H -4-OH-TAM-binding as studied in seven breast cancer samples with varying ER and AEBS_{tot} content (0-390 fmol ER/mg protein and 83-640 fmol AEBS_{tot} /mg protein).

This finding, and the observation that both TAM and 4-OH-TAM at 100- and 10,000-fold excesses gave almost the same inhibition of ^3H -TAM and ^3H -estradiol binding, respectively, suggest that ^3H -TAM and ^3H -4-OH-TAM bind to the same binding sites ($\text{AEBS}_{\text{spec}}$ and ER) in human breast cancer samples. This assumption was further supported by the observation (see below), that a 1000-fold excess of TAM and 4-OH-TAM also gave an identical inhibition of the binding of ^3H -4-OH-TAM.

Effects of excess estradiol on ^3H -4-OH-TAM binding. The study of the relative binding affinities to ER (see above) has shown that 4-OH-TAM has a much higher affinity to ER, compared with TAM. Therefore, it was investigated whether the inhibition of a 100-fold excess of estradiol on ^3H -4-OH-

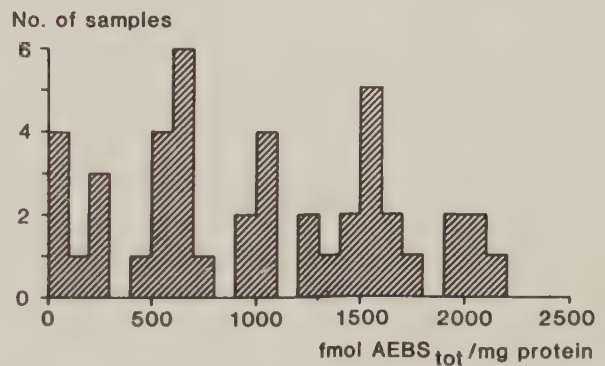


Figure 1. Frequency distribution of AEBS_{tot} concentrations of 44 breast cancer samples.

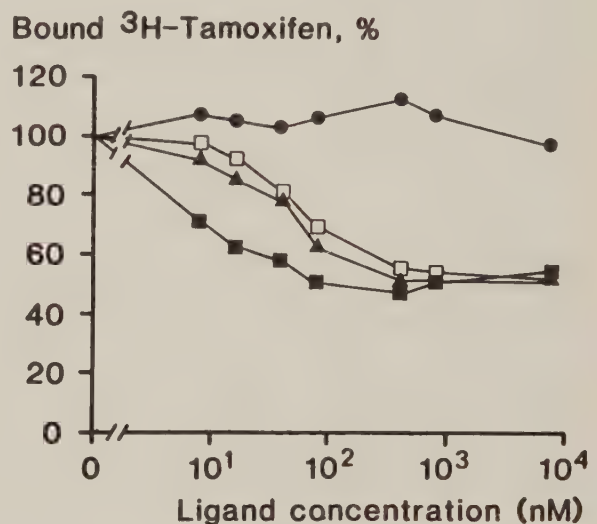


Figure 2. Competition for ^3H -TAM binding sites in 20,000xg supernatants from ER negative human breast cancer biopsy homogenates. Samples were incubated with 8.0 nM ^3H -TAM and with increasing concentrations (0-8.0 μM) of non-radioactive estradiol ($\bullet$), TAM ($\blacksquare$), 4-OH-TAM ($\blacktriangle$) or N-d-Me-TAM ($\square$) at 0°C for two hours. The data in the figure represent the mean values from three corresponding experiments and are presented as per cent total bound ^3H -TAM versus $^{10}\log$ ligand concentration.

TAM binding was better correlated with ER content in the same sample than when ^3H -TAM was the radioactive tracer. A study of 28 samples gave a correlation coefficient of 0.74 ($p<0.001$) between estradiol inhibition on ^3H -4-OH-TAM binding and ER content when all of the samples were included. In contrast to the findings above concerning estradiol inhibition on ^3H -TAM binding, this correlation persisted down to an ER/ AEBS_{tot} ratio of 0.01, which was found in 15 samples ($r_s=0.95$; $p<0.001$). The remaining 13 samples (ratio <0.01) showed no correlation ($r_s=-0.42$; $p=0.076$). It should be emphasized, however, that only three of these 13 samples gave contradictory results, e.g. high ER contents

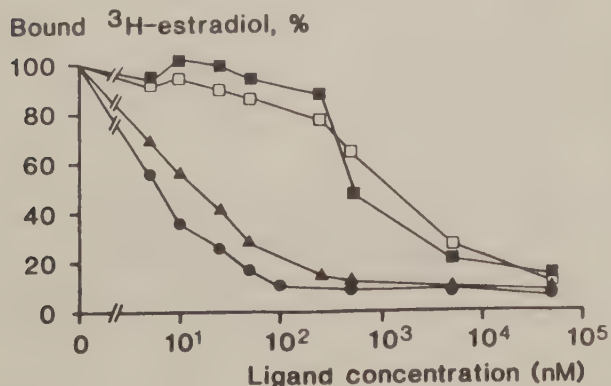


Figure 3. Competition for ^3H -estradiol binding sites in 20,000xg supernatants from ER positive human breast cancer biopsy homogenates. Samples were incubated with 5.0 nM ^3H -estradiol and with increasing concentrations (0-50 μM) of non-radioactive estradiol ($\bullet$), TAM ($\blacksquare$), 4-OH-TAM ($\blacktriangle$) or N-d-Me-TAM ($\square$) at 0°C for two hours. The data in the figure represent the mean values from five corresponding experiments and are presented as per cent total bound ^3H -estradiol versus $^{10}\log$ ligand concentration.

(110-150 fmol/mg protein) when measured from estradiol inhibition on ^3H -4-OH-TAM binding, but very low or non-detectable ER levels as determined with the IF technique. The inhibition in all of the three samples was furthermore less than 10 per cent, indicating no real inhibition, but only methodological uncertainty. The remaining ten samples gave low or non-detectable ER levels regardless of the method used. According to these findings, it is therefore suggested that ^3H -4-OH-TAM is a better choice of ligand than ^3H -TAM, when measuring AEBS_{spec}.

AEBS_{spec} concentration, K_d -value, correlation with ER and PgR. The concentration of AEBS_{spec} has been calculated in the 28 samples above by subtracting estradiol inhibition on ^3H -4-OH-TAM binding (e.g. the amount of ^3H -4-OH-TAM, which binds to ER) from the total ^3H -4-OH-TAM binding. The AEBS_{spec} concentration values ranged between 65-2,400 fmol/mg protein (mean $\pm$ S.D. = 860 ± 590), which should be compared with 120 ± 170 and 190 ± 330 , being the corresponding ER and PgR concentration values. The K_d -values for AEBS_{spec} varied between 0.45 and 4.5 nM (2.7 ± 1.7 nM). When comparing the concentration of AEBS_{spec} with ER and PgR content, correlation coefficients of 0.34 ($p=0.039$) and 0.16 ($p=0.25$) were obtained. The r_s -value of the correlation between AEBS_{spec} and ER content was 0.28 ($p=0.084$) when only ER positive samples were included ($n=26$).

RBA of estradiol and antiestrogens to the binding sites of ^3H -4-OH-TAM. The inhibition by an excess of estradiol, TAM, 4-OH-TAM and N-d-Me-TAM on the binding of ^3H -4-OH-TAM was studied in six ER negative samples, which means that only the binding to AEBS_{spec} was studied.

In agreement with the corresponding ^3H -TAM study (Figure 2), no influence from an excess of unlabelled estradiol was found. As can also be seen in Figure 4, the RBA of TAM, 4-OH-TAM and N-d-Me-TAM fell in the same order as when ^3H -TAM was the radioactive ligand (Figure 2), - with calculated RBA-values of 100%, 67% (60-74%) and 25% (11-69%), respectively. When, in the same way, five samples with considerable amounts of ER (>150 fmol ER/mg protein) were examined, an excess of non-radioactive estradiol did, in contrast to the ER negative cases above, inhibit ^3H -4-OH-TAM binding (Figure 5). 4-OH-TAM was here found to be the most potent inhibitor of ^3H -4-OH-TAM binding. In these experiments, the RBA-values of TAM and N-d-Me-TAM, as compared with 4-OH-TAM (100%), were 41% (20-76%) and 9.8% (5.1-14%), respectively.

Discussion

Treatment with antiestrogens, particularly tamoxifen (TAM), is at present the treatment of choice in breast cancer patients with disseminated disease and with ER positive tumour samples. Most of the anti-tumoural properties of TAM have been explained by an interference with ER (2). The finding of a specific antiestrogen binding site (AEBS_{spec}), distinct from ER, in human breast cancer samples is interesting, and such a binding site may contribute to the therapeutic effects of antiestrogens (5, 7, 10, 13).

The present study provides further support for the existence of AEBS_{spec} in human breast cancer. In contrast to earlier findings in breast cancer biopsies, where AEBS_{spec} was only found in ER positive samples (5, 7), AEBS_{spec} has in our investigation been found in both ER positive and ER negative biopsy samples. Thus, eight of nine ER negative samples contained considerable amounts of AEBS_{spec}, and in general only a very weak correlation between AEBS_{spec} and ER, whereas no correlation with PgR was found. In agreement with our results, a recent study was also able to demonstrate AEBS_{spec} in both ER positive and ER negative breast cancer biopsy samples (16). In contrast to steroid receptors, AEBS_{spec} was in the present study found in almost every breast cancer sample. The mean concentration of AEBS_{spec} was about 5-10 times that of ER and PgR. This investigation, as well as others (5, 8, 12), also clearly demonstrates that TAM and its main metabolites, 4-OH-TAM and N-d-Me-TAM, bind both to ER and AEBS_{spec}, although with different affinities. In contrast, estradiol binds only to ER. The data presented here show that TAM, compared to 4-OH-TAM and N-d-Me-TAM, has the strongest affinity to AEBS_{spec} after an incubation time of two hours. When the incubation time was prolonged to 20 hours no difference between the RBA-values of TAM and 4-OH-TAM could be demonstrated. These conclusions were drawn from experiments using ER negative samples, and either ^3H -TAM or ^3H -4-OH-TAM as the radioactive ligand. On the contrary, 4-OH-TAM was, in comparison with TAM and N-d-Me-

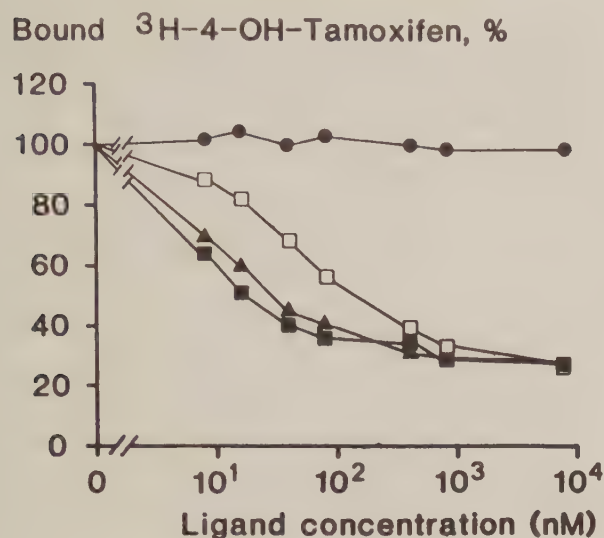


Figure 4. Competition for ^3H -4-OH-TAM binding sites in 20,000xg supernatants from ER negative human breast cancer biopsy homogenates. Samples were incubated with 8.0 nM ^3H -4-OH-TAM and with increasing concentrations (0–8.0 μM) of non-radioactive estradiol (●), TAM (■), 4-OH-TAM (▲) or N-d-Me-TAM (□) at 0°C for two hours. The data in the figure represent the mean values from six corresponding experiments and are presented as per cent total bound ^3H -4-OH-TAM versus $^{10}\log$ ligand concentration.

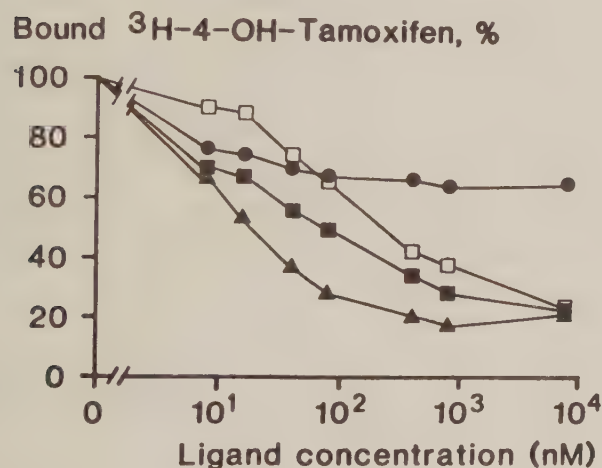


Figure 5. Competition for ^3H -4-OH-TAM binding sites in 20,000xg supernatants from ER positive (>150 fmol/mg protein) human breast cancer biopsy homogenates. Samples were incubated with 8.0 nM ^3H -4-OH-TAM and with increasing concentrations (0–8.0 μM) of non-radioactive estradiol (●), TAM (■), 4-OH-TAM (▲) or N-d-Me-TAM (□) at 0°C for two hours. The data in the figure represent the mean values from five corresponding experiments and are presented as per cent total bound ^3H -4-OH-TAM versus $^{10}\log$ ligand concentration.

sites of ^3H -4-OH-TAM in five samples with considerable amounts of ER (>150 fmol ER/mg protein).

When examining the influence of estradiol on ^3H -TAM binding, one would expect that excess non-radioactive estradiol, at least, would displace the fraction of ^3H -TAM, which binds to ER. This replacement only demonstrated a tendency to correlation with ER content in the few (6/43) samples with comparatively high ER/AEBS_{tot} ratios (≥ 0.20 , $r_s=0.66$; $p=0.078$). No correlation was found in samples where ER/AEBS_{tot} ratios were less than 0.20. One reason for this lack of correlation may be that an incubation time of two hours is too short for ^3H -TAM to reach equilibrium with ER. In control experiments, however, a longer incubation time (20 hours) has been compared with the shorter (2 hours), without resulting in any significant difference concerning this correlation (18 experiments, data not shown). This is not in conflict with the findings of Gulino *et al*⁽⁶⁾, who earlier have shown that maximal binding of ^3H -TAM was attained between one and two hours for both ER and AEBS_{spec} in fetal uterus cytosol of the guinea pig and remained stable for up to 18 hours. Objection may also be raised against the choice of isoelectric focusing instead of the DCC method with Scatchard analysis as the reference method for ER content. This argument seems, however, not to be valid, since an earlier investigation at our laboratory has shown that a comparison between IF and DCC for the measurement of ER gave a very strong correlation (17). A further reason for the lack of correlation between estradiol inhibition on ^3H -TAM binding and ER content in the same samples, as measured with IF, may be the low affinity of TAM to ER. The latter explanation is supported by the finding, that the inhibition of an excess of unlabelled estradiol on the binding of ^3H -4-OH-TAM, which has a much higher affinity to ER than TAM, gave a better correlation ($r_s=0.95$) with ER content in samples with much lower ER/AEBS_{tot} ratios (≥ 0.01) than in the cases when ^3H -TAM was used as the ligand. In order to measure AEBS_{spec} alone, it therefore seems preferable to use ^3H -4-OH-TAM as the radioactive ligand.

The existence of AEBS_{spec} in almost every one of the breast cancer samples demonstrated in this study, and the wide distribution to other organs (12, 18), confirmed in our laboratory (e.g. ovarian and endometrial carcinoma, in normal liver and uterine tissue from rabbit (data not shown)), makes it unlikely that AEBS_{spec} is directly involved in the estrogen antagonism of antiestrogens (12). Some investigators have suggested that AEBS_{spec} may act in a more passive way by altering the distribution volume of TAM (12). The wide range of AEBS_{spec} concentrations in breast cancer biopsy samples found in this work may possibly play a role in controlling the intracellular antiestrogen concentration and consequently, the amount of antiestrogens available for ER. A large variation in the concentration of TAM and its metabolites has also been found between different breast cancer patients, both in the tumour and plasma samples (19, 20). In DMBA-induced mammary tumours in rat, a correla-

TAM, shown to have the strongest affinity to ER, which would explain why 4-OH-TAM, compared with TAM, shows the strongest competition for the total number of binding

tion between response to TAM treatment and the cytosol concentration of these antiestrogenic compounds – at levels greatly exceeding their binding to ER – has been found (21). The finding that TAM binds to $AEBS_{spec}$ with a higher affinity than to ER suggests, however, that the concentration of $AEBS_{spec}$ might also in a negative way influence the amount of TAM, which is available for ER. This would perhaps be especially pronounced in cases with high $AEBS_{spec}/ER$ ratios.

Several investigators have not been able to show any correlation between $AEBS_{spec}$ and the growth-inhibiting effect of TAM in breast cancer cell-lines (8, 9). Faye *et al*⁽¹³⁾, however, have reported, that TAM only had effect in a breast cancer cell-line, which contained both ER and $AEBS_{spec}$, while it was ineffective in an ER positive cell-line lacking $AEBS_{spec}$. In addition, Saez *et al*⁽¹⁰⁾ demonstrated a positive effect of TAM in a breast cancer cell-line with $AEBS_{spec}$ but without ER. Although the data are limited, $AEBS_{spec}$ may thus be of significance for TAM effects, at least *in vitro*. No work in a clinical breast cancer material has as yet been performed, however.

In conclusion, this investigation has confirmed the presence of a specific binding site ($AEBS_{spec}$) for TAM and its main metabolites (4-OH-TAM and N-d-Me-TAM) in human breast cancer biopsies. It has further been shown that the concentration of $AEBS_{spec}$ was very weakly correlated to ER content, whereas no correlation to PgR content was found. Our investigation has also confirmed the finding that 4-OH-TAM compared with TAM has a much stronger affinity to ER. This may be one reason why the inhibition of excessive estradiol on 3H -4-OH-TAM binding was better correlated with ER content in the same sample than if 3H -TAM was the radioactive tracer. It is therefore suggested that 3H -4-OH-TAM compared with 3H -TAM is a better ligand for measuring $AEBS_{spec}$. Taking the wide range of $AEBS_{spec}$ concentration values found in the present study into consideration, it should be worthwhile to perform further work for establishing possible correlations between the amount of $AEBS_{spec}$ in relation to ER content and the clinical effects of TAM in a breast cancer material. Such a study would perhaps also help to explain why certain breast cancer patients with receptor positive tumours fail to respond to TAM treatment, as well as the reason why some with receptor negatives occasionally do so.

Acknowledgements

The present investigation was in part supported by grants from the Swedish Cancer Society, the Swedish Medical Research Council, John and Augusta Persson's Foundation, and from the Medical Faculty of the University of Lund, Sweden. The authors wish to express their gratitude to Ulla Johansson and Gunilla Sellberg, for skilful technical assistance, and to Bo Gullberg, for statistical advice.

References

- Mouridsen H, Palshof T, Patterson J, and Battersby L: Tamoxifen in advanced breast cancer. *Cancer Treat Rev* 5: 131-141, 1978.
- Katzenellenbogen BS, Bhakoo HS, Ferguson ER, Lan NC, Tatee T, Tsai TS, and Katzenellenbogen JA: Estrogen and antiestrogen action in reproductive tissues and tumours. *Recent Progress in Hormone Research* 35: 259-300, 1979.
- Cowan K and Lippman M: Steroid receptors in breast cancer. *Arch Intern Med* 142: 363-366, Feb 1982.
- Sutherland RL and Foo MS: Differential binding of antiestrogens by rat uterine and chick oviduct cytosol. *Biochem Biophys Res Commun* 91: 183-191, 1979.
- Sutherland RL, Murphy LC, Foo MS, Green MD, Whybourne AM, and Krozowski ZS: High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. *Nature* 288: 273-275, 1980.
- Gulino A and Pasqualini JR: Heterogeneity of binding sites for tamoxifen and tamoxifen derivatives in estrogen target and nontarget fetal organs of guinea pig. *Cancer Research* 42: 1913-1921, 1982.
- Sutherland RL and Murphy LC: The binding of tamoxifen to human mammary carcinoma cytosol. *Europ J Cancer* 16: 1141-1148, 1980.
- Miller MA and Katzenellenbogen BS: Characterization and quantitation of antiestrogen binding sites in estrogen receptor-positive and -negative human breast cancer cell lines. *Cancer Research* 43: 3094-3100, 1983.
- Wakeling AE, Valcaccia B, Newbould E, and Green LR: Non-steroidal antioestrogens - receptor binding and biological response in rat uterus, rat mammary carcinoma and human breast cancer cells. *J Steroid Biochem* 20 (1): 111-120, 1984.
- Saez S and Chouvet C: Evidence for high affinity 3H -tamoxifen cytosol binding protein (s) in a human breast cancer cell line MDA-MB 231 devoid of estrogen receptors. *Proc Am Assoc Cancer Res* 23: 710, 1983.
- Watts CKW, Murphy LC, and Sutherland RL: Microsomal binding sites for nonsteroidal anti-estrogens in MCF 7 human mammary carcinoma cells. Demonstration of high affinity and narrow specificity for basic ether derivatives of triphenylethylene. *Biol Chem* 259 (7): 4223-4229, 1984.
- Sudo K, Monsma FJ, and Katzenellenbogen BS: Antiestrogen binding sites distinct from the estrogen receptor: subcellular localization, ligand specificity, and distribution in tissues of the rat. *Endocrinology* 112 (2): 425-434, 1983.
- Faye J-C, Jozan S, Redeuilh G, Baulieu E-E, and Bayard F: Physicochemical and genetic evidence for specific antiestrogen binding sites. *Proc Natl Acad Sci* 80: 3158-3162, 1983.
- Norgren A, Borg Å, Fernö M, Johansson U, Lindahl B, and Tsiobanelis K: Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer. *Anticancer Research* 2: 315-320, 1982.
- Lowry OH, Rosebrough NJ, Farr L, and Randall RJ: Protein measurement with the folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
- Chouvet C and Saez S: High affinity tamoxifen cytosol binding site (s) in human breast cancer biopsies and cell-lines devoid of estrogen receptors. *International Congress in Endocrinology of the Breast (Basic and Clinical aspects)*, abstract 68, September 1984, Torino, Italy.
- Fernö M, Borg Å, and Norgren A: A comparison of two steroid receptor assays in breast cancer: dextran coated charcoal and isoelectric focusing. *Anticancer Research* 3: 243-246, 1983.
- Kon OL: An antiestrogen-binding protein in human tissues. *J Biol Chem* 258 (5): 3173-3177, 1983.
- Daniel P, Gaskell SJ, Bishop H, Campbell C, and Nicholson RI: Determination of tamoxifen and biologically active metabolites in human breast tumours and plasma. *Europ J Cancer Clin Oncol* 17 (11): 1183-1189, 1981.
- Jordan VC: Review. Metabolites of tamoxifen in animals and man: identification, pharmacology, and significance. *Breast Cancer Research and Treatment* 2: 123-138, 1982.
- Daniel CP, Gaskell SJ, and Nicholson RI: The measurement of tamoxifen and metabolites in the rat and relationship to the response of DMBA-induced mammary tumours. *Europ J Cancer Clin Oncol* 20 (1): 137-143, 1984.

Received January 17, 1985

**ESTRAMUSTINE BINDING SITE
IN HUMAN BREAST CANCER BIOPSY SAMPLES**

**Its Relation to Estrogen and Progesterone Receptor Levels,
Age and Menopausal Status**

Mårten Fernö, Åke Borg & Ingrid Idvall

**Departments of Oncology and Cytodiagnostics
University Hospital
S-221 85 LUND
Sweden**

ABSTRACT

Estramustine is the cytotoxic metabolite of estramustine phosphate (Estracyt®), which is used in the treatment of prostatic carcinoma. With isoelectric focusing in polyacrylamide gels an estramustine binding site (EMBS) at pH 4.8-4.9 was demonstrated in 74 of 306 (24%) breast cancer biopsy samples. Presence of EMBS was significantly ($p < 0.001$) correlated with negative or low estrogen and progesterone receptor values. EMBS positivity was found in 31% of the samples from pre- and peri-menopausal patients and in 22% of the samples from postmenopausal patients. If patients were instead divided into different age groups, EMBS positivity was most frequent in samples from patients between 50 and 59 years of age (42%). With increasing age the percentage of EMBS positivity fell successively. Only 3 of 40 (7.5%) samples from patients 80 years of age or older were EMBS positive. For patients under 50 years, no difference with respect to EMBS positivity between age groups could be demonstrated. Thus, the changes in the hormonal milieu during and after menopause seem to affect the probability of finding EMBS in tumor samples. The possible value of EMBS determinations in breast cancer tissue specimens for the selection of those patients that will respond to Estracyt® therapy should be evaluated.

INTRODUCTION

Estramustine phosphate (EMP, Estracyt®) is a nornitrogen mustard derivate of estradiol-17 β -phosphate, which has been used since 1966 for the treatment of prostatic carcinoma (1). In the body EMP is rapidly dephosphorylated to estramustine, which is oxidized to a higher extent to the estrone counterpart of estramustine, estromustine (2). Estramustine and most probably estromustine are believed to exert cytotoxic activity through interaction with microtubule associated proteins, inducing mitotic arrest of cells in metaphase (3,4) and by interaction with the nuclear protein matrix causing cell death at the level of DNA replication (5).

A route for preserving the prostatic tumor cells with cytotoxic EMP metabolites has been suggested as a protein, binding estramustine and estromustine specifically and with high affinity, was discovered in the prostate (6,7). This protein was called "estramustine-binding protein" (EMBP) due to the unique binding affinity for these ligands. Though the biological function of this protein is not fully understood, proteins in the rat ventral prostate that most probably are identical to EMBP have also been described by others: " α -protein" (8); "prostatic binding protein" (9); "prostatein" (10); "prostatic secretion protein" (11).

A few clinical trials with EMP in the therapy of other malignancies (e.g. malignant melanoma, renal cell carcinoma and breast cancer) have also been reported (12,13,14,15,16). In case of breast cancer, three investigations have shown response rates in 17/44, 2/16 and 2/34, respectively, of patients with advanced disease (14,15,16).

Trying to explain why only certain breast cancer patients respond to EMP therapy, the binding of estramustine to breast cancer biopsy samples has been investigated in order to find a binding site, similar to EMBP in the prostatic tissue. This study has been performed in parallel to the routine measurement of estrogen and progesterone receptors in breast cancer samples.

MATERIAL AND METHODS

Tissue Material

Estramustine binding site (EMBS) and estrogen receptor (ER) have been examined in 306 and progesterone receptor (PgR) in 294 consecutive breast cancer biopsy samples. Eightythree of the patients were classified as pre- or peri-menopausal (less than five years after the last regular menstrual bleeding) and 223 as postmenopausal (more than five years after the last regular menstrual bleeding). Biopsy material was obtained at operation and was freed from fat, blood clots etc., minced and then kept for not more than two weeks at either -70°C or in liquid nitrogen until analysis.

Chemicals and Ligands

All chemicals were of analytical grade. $(2,4,6,7-^3\text{H})$ Estradiol (91-93 Ci/mmol) was obtained from Amersham International, Buckinghamshire, England. (^3H) Promegestone (^3H -R 5020, 87 Ci/mmol) and non-radioactive promegestone were purchased from New England Nuclear, Boston, Ma, U.S.A. Non-radioactive estradiol and $(2,4,6,7-^3\text{H})$ estramustine (104 Ci/mmol) were kindly supplied by AB LEO Research Laboratories, Helsingborg, Sweden.

Analytical procedures

Tumor tissue was homogenized with an all-glass Potter-Elvehjem homogenizer in TEM-buffer (Tris-HCl 10 mM, EDTA 1.5 mM, mercaptoethanol 10 mM, pH 7.4) at 0°C . Centrifugation of the homogenate was carried out at $20,000 \times g$ for 10 minutes. The concentration of proteins was adjusted to 1-3 mg/ml and aliquots were analyzed for ER, PgR and EMBS as described below.

ER was measured with isoelectric focusing in polyacrylamide gels (IF) and PgR with the multiple point dextran-coated charcoal (DCC) method and Scatchard analysis as described previously (17).

Analysis of EMBS was performed with the IF technique according to the following brief description: (^3H) Estramustine dissolved in absolute ethanol was added to the $20,000 \times g$ supernatant to a final concentration of 7.5 nM (final ethanol concentration was 3.2%). After 2 hours incubation at 0°C , the sample was treated with DCC (dextran:charcoal 0.03:0.3 (mg/ml), final concentrations) for 1 minute followed by a centrifugation

at 3,000 x g for 10 minutes. The supernatant was applied in polyacrylamide gels and run as described previously (17). The radioactivity was measured over the pH-range 3.5-9.5.

Human serum albumin (HSA) has been reported to interfere with the binding assay for estramustine in human prostatic samples (7). Therefore, for control, HSA was labelled with (^3H)estramustine and analyzed by the IF technique above.

The concentrations of ER and PgR were expressed as femto(f)mol specifically bound (^3H)estradiol (ER) and (^3H)R 5020 (PgR) per mg protein. Concentrations below 10 fmol ER/mg protein and 30 fmol PgR/mg protein were considered negative or low (ER- and PgR-). The amount of EMBS was only stated as positive or negative (EMBS+ or EMBS-). The protein concentration was measured according to the method described by Lowry et al. (18).

Statistics

Evaluation of differences was made with chi-square(χ^2)-analysis and p-values above 0.05 were considered as not having statistical significance.

RESULTS

Representative chromatograms from EMBS assays are shown in Fig. 1a-c. Fig. 1a demonstrates a distinct peak of (^3H)estramustine radioactivity at pH 4.8-4.9, whereas in Fig. 1c significant radioactivity at this pH is absent. The chromatogram in Fig. 1b shows an intermediate appearance with no obvious peak at the appropriate pH, but a plateau of radioactivity clearly distinguished from the background activity as seen in Fig. 1c. In control experiments it was shown that samples with a distinct radioactive peak as in Fig. 1a gave the same type of chromatograms as shown in Fig. 1b when diluted with an EMBS- sample and with no major change in protein concentration (data not shown, four experiments). Due to these observa-

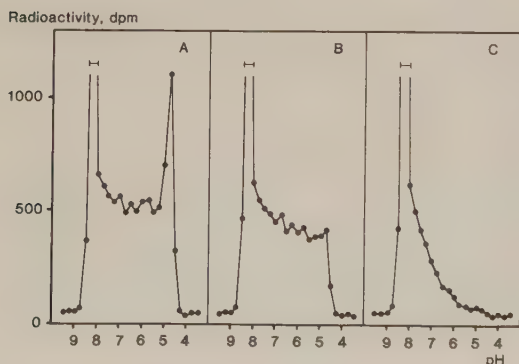


Figure 1.
Representative chromatograms from EMBS assays with isoelectric focusing in polyacrylamide gels illustrating:
a/ a distinct peak of (^3H)estramustine radioactivity at pH 4.8-4.9.
b/ an (^3H)estramustine radioactivity plateau extending to pH 4.8-4.9, but without any distinct peak as seen in Fig. 1a.
c/ a chromatogram with no activity besides background radioactivity at the appropriate pH.
Samples with chromatograms as in a/ and b/ were considered as EMBS positive (EMBS+), whereas samples with an appearance as in c/ were stated as negative (EMBS-).

tions, samples with radioactive profiles as Fig. 1a and 1b are referred to as EMBS+, whereas samples with the appearance as in Fig. 1c consequently are classified as EMBS-.

HSA labelled with (³H)estramustine was focused around pH 5.2 and therefore no interference with the qualitative evaluation of the EMBS assay was obtained (data not shown).

In the present investigation 74/306 (24%) breast cancer samples were EMBS+ according to the definitions above. In Fig. 2 and 3 ER and PgR concentrations are plotted against EMBS+ and EMBS- samples. Fig. 2 shows that EMBS+ samples have lower mean ($\pm$ SD) ER values (20 ± 35 fmol ER/mg protein) compared with EMBS- samples (160 ± 220 fmol ER/mg protein). The corresponding figures for PgR were 57 ± 150 and 200 ± 340 , respectively (Fig. 3). Fig. 2 and 3 also show, that EMBS+ was overrepresented in ER- and PgR- samples. Thus, in this study, 47/119 (39%) ER- samples contained EMBS, whereas only 27/187 (14%) ER positive (ER+) samples were EMBS+

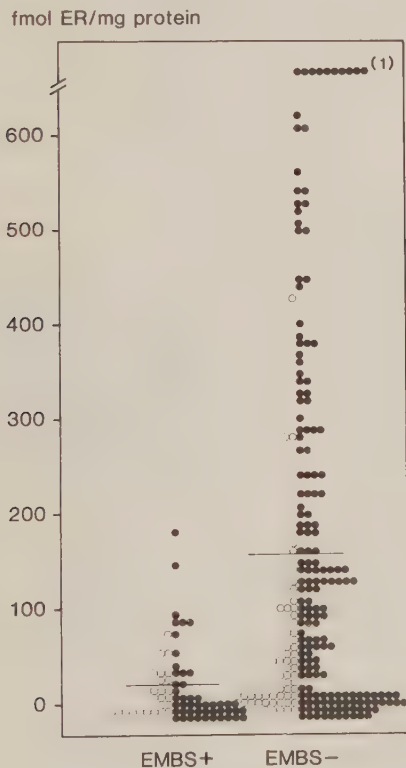


Figure 2.
Distribution of ER concentrations (fmol/mg protein) in EMBS+ and EMBS- samples. Mean ($\pm$ SD) ER values in the EMBS+ group (n=74) were 20 ± 35 fmol/mg protein. In the EMBS- group (n=232) the corresponding figures were 160 ± 220 fmol/mg protein. Mean values are marked with arrows in the figure. Samples from pre- and peri-menopausal patients are marked with O, whereas samples from postmenopausal patients are marked with ●.
(1) 700-1,300 fmol/mg protein.

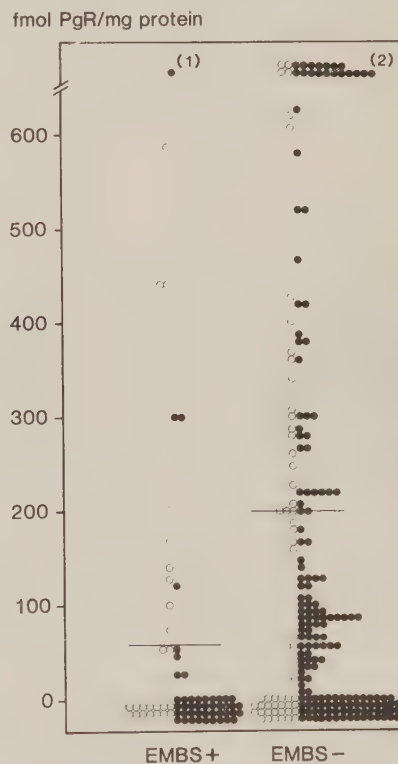


Figure 3.
Distribution of PgR concentrations (fmol/mg protein) in EMBS+ and EMBS- samples. Mean ($\pm$ SD) PgR values in the EMBS+ group (n=72) were 57 ± 150 fmol/mg protein. In the EMBS- group (n=222) the corresponding figures were 200 ± 340 fmol/mg protein. Mean values are marked with arrows in the figure. Samples from pre- and peri-menopausal patients are marked with O, whereas samples from postmenopausal patients are marked with ●.
(1) 850 fmol/mg protein.
(2) 660-2,300 fmol/mg protein.

($p < 0.001$). The corresponding figures concerning PgR were 55/147 (37%; PgR- samples) and 17/147 (12%; PgR positive (PgR+) samples), respectively ($p < 0.001$). The proportion of EMBS+ in samples from pre- and peri-menopausal patients was 26/83 (31%), whereas 48/223 (22%) samples from postmenopausal patients were EMBS+.

Fig. 4 shows the distribution of EMBS+ samples with age. The highest frequency of EMBS+ (42%) was found in samples from patients between 50 and 59 years of age. As this group is heterogeneous with regard to menopausal status, the frequency of EMBS+ in this group was studied for both pre/peri- and post-menopausal patients. Ten of 22 (45%) of the tumor samples from pre- and peri-menopausal patients were then found to be EMBS+. The corresponding figures for postmenopausals were 15/37 (41%). One of 43 patients between 40 and 49 years of age was postmenopausal, which means that the actual percentage of EMBS+ samples from pre- and peri-menopausals in this age group was 26 (11/42). Five of 19 (26%) samples from patients less than 40 years were EMBS+. After the EMBS+ peak between 50 and 59 years the frequency fell with increasing age and for samples from patients 80 years of age or older only 3/40 (7.5%) were EMBS+.

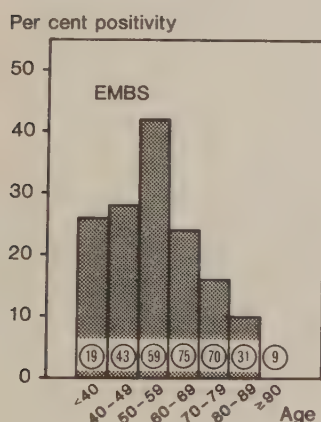


Figure 4.
Per cent EMBS positive (EMBS+; for explanation see Fig. 1a-c) breast cancer samples in relation to patient age at operation. The number of samples in each age group is indicated in the bars.

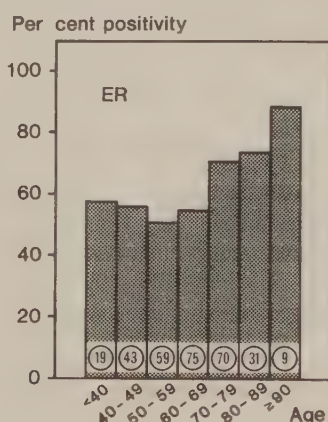


Figure 5.
Per cent ER positive (>10 fmol/mg protein) breast cancer samples in relation to patient age at operation. The number of samples in each age group is indicated in the bars.

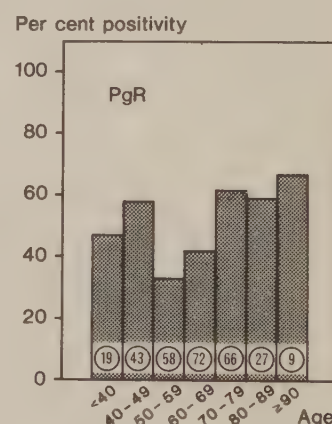


Figure 6.
Per cent PgR positive (>30 fmol/mg protein) breast cancer samples in relation to patient age at operation. The number of samples in each age group is indicated in the bars.

Fig. 5 and 6 show the age distribution patterns for ER+ and PgR+ samples, respectively. For ER+ and PgR+ samples, qualitatively different age distribution profiles as compared to EMBS+ samples were obtained. While a peak value for EMBS+ was demonstrated between 50 and 59 years, the frequencies for ER+ and PgR+ were the lowest in this group (51% and 33%, respectively). When dividing the patients in this age group into menopausal status, ER+ was found in 55% of the samples from pre- and peri-menopausal status, ER+ was found in 55% of the samples from pre- and peri-menopausal status, ER+ was found in 55% of the samples from pre- and peri-menopausal status.

pausal patients and in 49% of the samples from postmenopausal patients. The corresponding figures for PgR+ samples were 27% and 36%, respectively. For patients older than 59 years an increase in receptor positivity with increasing age was obtained, which contrasted with the decrease in EMBS+ samples in older patients. When examining the distribution of EMBS both in relation to menopausal and receptor status, a statistically significant correlation between EMBS+ and low or negative receptor concentrations was found only for postmenopausal patients (Table 1 and 2).

Table 1.
Correlations between EMBS and ER status in breast cancer tumor samples from pre/perimenopausal and postmenopausal patients. The figures in the Table represent the number of samples and statistical evaluations were made with chi-square analysis. Cut-off level for ER was 10 fmol/mg protein. For EMBS+/- criteria see Fig 1.

	Pre/perimenopausal		Postmenopausal	
	EMBS+	EMBS-	EMBS+	EMBS-
ER+	12	35	15	125
ER-	14	22	33	50
	p=0.19 NS		p<0.001	

Table 2.
Correlations between EMBS and PgR status in breast cancer tumor samples from pre/perimenopausal and postmenopausal patients. The figures in the Table represent the number of samples and statistical evaluations were made with chi-square analysis. Cut-off level for PgR was 30 fmol/mg protein. For EMBS+/- criteria see Fig 1.

	Pre/perimenopausal		Postmenopausal	
	EMBS+	EMBS-	EMBS+	EMBS-
PgR+	11	30	6	100
PgR-	15	27	40	65
	p=0.38 NS		p<0.001	

DISCUSSION

The present investigation has demonstrated an estramustine binding site (EMBS) in 74/306 (24%) consecutive breast cancer biopsy samples. EMBS+ was found to be strongly correlated with ER- and PgR-. When studying the frequency of EMBS+ in relation to the patient age a EMBS+ peak value was found in samples from patients between 50 and 59 years of age. Forty-two per cent of the samples from patients in this group were EMBS+, which was markedly higher than the corresponding figures for younger as well as older patients. It should be mentioned, that in the age group between 50 and 59, no obvious difference in the frequency of EMBS+ was found between pre/perimenopausal and postmenopausal patients (45% and 41%, respectively). However, since in this study, the perimenopausal period has been defined as up to five years after the last regular menstrual bleeding, one can assume that patients from the age group between 50 and 59 years in general should have a hormonal status representative for menopausal or postmenopausal women. Samples from older postmenopausal patients showed a successively decreasing per cent of EMBS+ and it is worth mentioning that none of 29 tumor samples from patients older than 82 years was EMBS+. With regard to ER and PgR status the opposite pattern was obtained. Samples from older postmenopausal patients were more often receptor positive than samples from younger postmenopausal patients. Also, in contrast to

the EMBS pattern, the lowest frequency of receptor positivity was found for samples from the age group between 50 and 59 years. For pre- and peri-menopausal younger than 50 years only slight differences in receptor status between age groups could be demonstrated. When EMBS status was correlated with receptor status, a statistical significant correlation between EMBS+ and low or negative receptor values could only be established for postmenopausal patients. However, when comparing the mean receptor concentrations for EMBS+ samples with the corresponding values for EMBS- samples a difference was found which was independent of menopausal status. The mean ER and PgR concentrations were significantly lower for EMBS+ samples as compared with EMBS- samples from both pre/peri- and post-menopausal patients (data not shown).

It is possible that the observed differences in EMBS and receptor status may reflect the changes in the hormonal milieu which appear during and after the menopause. The menopausal ovarian function is characterized by a fall in estrogen and progesterone secretion (19). Furthermore, it has been suggested that the primary function of the postmenopausal ovary is to secrete androgens and androgen precursors, which should contribute to a shift to a more androgenic milieu after the menopause (20). Such hormonal changes may be of relevance for the EMBS+ peak observed in the present work, as well as the high frequency of receptor negativity in samples from patients between 50 and 59 years. In this context it is also interesting that, in the ventral prostate from rat, EMBP was found to be dependent on androgenic stimuli (21,22). The concentration of EMBP showed marked changes as a function of age, ranging from very low levels in immature rats to more than two-hundred fold increased levels in adults. After castration the concentration of EMBP dropped to less than 10 per cent of precastration levels, but returned to normal levels after two weeks of androgen treatment (21). Further studies are needed in order to investigate a possible similarity between EMBP in the prostate and EMBS in breast cancer. Moreover, it would be of great interest to investigate a possible correlation between EMBS and androgens in breast cancer.

In this work no attempts have been made to quantitate the relative amount of EMBS. However, as indicated in Results, different concentrations of EMBS resulted in differences in the chromatograms (Fig. 1a and 1b). Furthermore, variations in EMBS concentration have also been indicated when studying the height of the radioactive peak in the samples with an appearance as in Fig. 1a. In this context it should also be mentioned that preliminary experiments at our laboratory have indicated that the presence of endogenous ligand(s) may be of importance for the quantification of EMBS. Treatment with dextran-coated charcoal, prior to incubation with (³H)estramustine have in some cases resulted in a higher

degree of binding activity (data not shown). The possible importance of these variations in EMBS content is indicated when correlations with receptor concentrations are performed. Thus, the fifty samples with a distinct peak of (³H)estramustine radioactivity had lower mean ER and PgR concentrations (11±18 and 21±49 fmol/mg protein, respectively) compared with the twenty-four samples with an intermediate appearance as in Fig. 1b (37±52 and 140±240 fmol/mg protein, respectively). As stated before, these groups, both considered to be EMBS+, had much lower mean receptor concentrations than EMBS- samples.

It is now well established that there is a positive correlation between receptor content and the effect of endocrine therapy in patients with disseminated breast cancer (23). Of patients with ER and PgR positive tumor samples, about 70% will respond to hormonal treatment, whereas less than 10% without significant amounts of receptors will respond to this therapy (24). Consequently, the latter group will usually be given cytotoxic drugs as first line therapy. For this group, however, it seems as if the study reported here should motivate a clinical investigation on the effects of estramustine phosphate in postmenopausal breast cancer patients with ER- and PgR- tumors. In such a study EMBS should also be measured in order to evaluate its significance for the clinical efficacy of Estracyt[®] in breast cancer treatment.

ACKNOWLEDGEMENTS

The present investigation was in part supported by grants from Swedish Cancer Society, the Swedish Society of Medical Sciences, John and Augusta Person's Foundation and from the Medical Faculty of the University of Lund, Sweden. The authors wish to express their gratitude to Per Björk and Jonas Müntzing for valuable discussions and criticisms and to Ulla Johansson and Gunilla Sellberg for skilful technical assistance.

REFERENCES

1. Edsmyr, F., Andersson, L., and Könyves, I. Estramustine phosphate (Estracyt): Experimental studies and clinical experience. In: Jacobi, G.H., and Hohenfeller, R. (eds.) *Prostate Cancer, International Perspectives in Urology* (Williams & Wilkins, Baltimore) 3:253-268, 1982.
2. Gunnarsson, P.O., Plym Forsell, G., Fritjofsson, A., and Norlén, B.J. Plasma concentrations of estramustine phosphate and its major metabolites in patients with prostatic carcinoma treated with different doses of estramustine phosphate (Estracyt®). *Scand. J. Urol. Nephrol.* 15: 201-206, 1981.
3. Hartley-Asp, B. Estramustine-induced mitotic arrest in two human prostatic carcinoma cell lines, DU 145 and PC-3. *The Prostate* Vol. 5, No. 1: 93-100, 1984.
4. Kanje, M., Deinum, J., Wallin, M., Ekström, P., Edström, A., and Hartley-Asp, B. Estramustine phosphate inhibits assembly of isolated brain microtubules and fast axonal transport. *Cancer Res.* In press.
5. Tew, K.D., Erickson, L.C., White, G., Wang, A.L., Schein, P.S., and Hartley-Asp, B. Cytotoxicity of estramustine, a steroid-nitrogen mustard derivative through non-DNA targets. *Mol. Pharmacol.*, 24: 324-328, 1983.
6. Forsgren, B., Björk, P., Carlström, K., Gustafsson, J.-Å., Pousette, A., and Högborg, B. Purification and distribution of a major protein in rat prostate that binds estramustine, a nitrogen mustard derivative of estradiol-17 β . *Proc. Natl. Acad. Sci. USA*, Vol. 76, No. 7: 3149-3153, 1979.
7. Björk, P., Forsgren, B., Gustafsson, J.-Å., Pousette, A., and Högborg, B. Partial characterization and "quantitation" of a human prostatic estramustine-binding protein. *Cancer Research* 42:1935-1942, 1982.
8. Liao, S., Tymoczko, J.L., Liang, T., Anderson, K.M., and Fang, S. Androgen receptors: 17 β -hydroxy-5 α -androstane-3-one and the translocation of a cytoplasmic protein to cell nuclei in prostate. *Adv. Biosci.*, 7: 155-163, 1971.
9. Heyns, W., and De Moor, P. Prostatic binding protein. A steroid binding protein secreted by rat prostate. *Eur. J. Biochem.*, 78:221-230, 1977.
10. Lea, O., Petrusz, P., and French, F. Prostatein. A major secretory protein of the rat ventral prostate. *J. Biol. Chem.*, Vol. 254, No. 13: 6196-6202, 1979.
11. Pousette, A., Björk, P., Carlström, K., Forsgren, B., Gustafsson, J.-Å., and Högborg, B. On the presence of "prostatic secretion protein" in different species. *Acta Chem. Scand.*, B 34 (2): 155-156, 1980.
12. Lopez, R., Karakousis, C.P., Didolkar, M.S., and Holyoke, E.D. Estramustine phosphate in the treatment of advanced malignant melanoma. *Cancer Treat. Rep.*, 62: 1329-1332, 1978.
13. Swanson, D.A., and Johnson, D.E. Estramustine phosphate (Emcyt) as treatment for metastatic renal carcinoma. *Urology*, 17 (4): 344-346, 1975.

14. Alexander,N.C., Hancock,A.K., Masood,M.B., Peet,B.G., Price,J.J., Turner,R.L., Stone,J., and Ward,A.J. Estracyt in advanced carcinoma of the breast: A Phase II Study. Clin. Radiol. 30:139-147, 1979.
15. Dawes,P.J.D.K. A pilot study of Estracyt in advanced breast cancer. Cancer Treat. Rep. Vol. 66, No. 3, 1982.
16. Groupe Europeen du Cancer du Sein. Essai clinique du phenol bis (2-chloroethyl) carbamate d'estradiol dans le cancer mammaire en phase avancee. Europ. J. Cancer, 5: 1-4, 1969.
17. Norgren,A., Borg,A., Fernö,M., Johansson,U., Lindahl,B., and Tsiobanellis,K. Improved method for assay of estradiol and progesterone receptors with special reference to breast cancer. Anticancer Research, 2: 315-320, 1982.
18. Lowry,O.H., Rosebrough, N.J., Farr,L., and Randall,R.J. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275, 1951.
19. Vermeulen,A. The hormonal activity of the postmenopausal ovary. J. Clin. Endocrin. Metab., Vol. 42, No. 2: 247-253, 1976.
20. Greenblatt,R.B., Colle,M.L., and Mahesh,V.B. Ovarian and adrenal steroid production in the postmenopausal woman. Obstetrics and Gynecology, Vol. 47, No. 4: 383-387, 1976.
21. Heyns,W., Van Damme,B., and De Moor,P. Secretion of prostatic binding protein by rat ventral prostate: Influence of age and androgen. Endocrinology, Vol. 103, No. 4: 1090-1095, 1978.
22. Pousette,A., Björk,P., Carlström,K., Forsgren,B., Högberg,B., and Gustafsson,J-A. Influence of sex hormones on prostatic secretion protein, a major protein in rat prostate. Cancer Research, 41, 688-690, 1981.
23. McGuire,W.L. An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. Hormones and Cancer, edited by S. Jacobello et al., Raven Press, New York, 337-343, 1980.
24. McGuire,W.L., and Clark,G.M. Progesterone receptors and human breast cancer; 'Wassink lecture' presented at the 3rd EORTC Breast Cancer Working Conference. Eur. J. Cancer Clin. Oncol. Vol. 19, No. 12: 1681-1685, 1983.

